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Sanitizing efficacy of an environmentally friendly combination hot tub product – eco3spa

A thesis submitted to Swansea University College of Engineering in fulfilment of the requirements for the degree of Master of Science by Research in Materials Engineering

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II. Abstract

Hot tub use is becoming increasingly popular in the UK. However, incorrect maintenance and cleaning can lead to the development of a vast variety of biofilms formed by microorganisms such as *Escherichia* coli and *Pseudomonas aeruginosa,* an opportunistic pathogen. The ease of proliferation and challenging removal of *P. aeruginosa* biofilms are of especially great importance to immunocompromised and elderly people, who can be vulnerable to such opportunistic infections.

The aim of this study was to analyze the efficacy of a newly developed, environmentally friendly water sanitation, biofilm prevention and removal combination product for the domestic hot tub sector. The product includes active oxygen which is combined with a water conditioner and surfactants whose activities were tested against planktonic growth and biofilm formation. *E. coli* K12 or *P. aeruginosa* PA01 cells were grown and biofilms established under static conditions at 37 and 25°C which are standard hot tub operational and down-time temperatures, respectively. A range of product concentrations and combinations were assessed over a hot tub-relevant pH range for determining their efficacy of inhibiting planktonic growth and biofilm prevention and removal properties.

Results indicated that in standard operating procedure conditions, active oxygen in the recommended concentration for use as well as up to ten times more dilute is effective at preventing planktonic growth and biofilm formation in both species across both temperatures and all pH levels tested. In addition, the combination with the water conditioner appeared to neither inhibit nor enhance active oxygen activity. Light and fluorescence microscopy suggested the active oxygen by itself or in combination with the conditioner may lead to viable-but-non-culturable cell formation.

This study has provided novel insights on the activity of active oxygen over a range of three pH and two temperatures, and the results generated may assist in the development of new antimicrobial or disinfecting formulations. Additionally, the industrial sponsor can now make scientifically substantiated claims about the sanitizing efficacy of their products and promote the use of an eco-friendly, non-toxic alternative to chlorine for the domestic hot tub sector.

III. DECLARATION

This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree.

Signed: 

Date: 20/04/21

This thesis is the result of my own investigations, except where otherwise stated. Other sources are acknowledged by footnotes giving explicit references. A bibliography is appended.

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I hereby give consent for my thesis, if accepted, to be available for photocopying and for inter-library loan, and for the title and summary to be made available to outside organisations.

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Abbreviation	Definition
BSA	Bovine serum albumin
c-di-GMP	cyclic dimeric guanosine monophosphate
CFU	colony forming units
CRP	Cyclic AMP (cAMP) receptor protein
DGC	diguanylate cyclase
EET	extracellular electron transfer
EPS	extracellular polymeric substances
GASP	Growth Advantage in Stationary Phase
HGT	horizontal gene transfer
INT	p-iodonitrotetrazolium violet
LB	luria-bertani
NaDCC	Sodium dichloroisocyanurate
OD	optical density
PBS	phosphate buffered saline
PCA	phenazine carboxylate
PCN	phenazine carboxamide
PGA	ß-1,6- *N*-acetyl-D-glucosamine polymer
PI	Propidium iodide
PIA	polysaccharide intercellular adhesion polymers
PMS	pentapotassium peroxymonosulfate
PTS	phosphoenolpyruvate: sugar phosphotransferase system
PYO	pyocyanin
QS	quorum sensing
RNAP	DNA-dependent RNA polymerase
SEM	standard error of the mean
SOP	standard operating procedures
TCA	Trichloroacetic acid
VBNC	viable but non-culturable

Chapter One: Introduction

1.1 Background information

Hot tubs are commonly used for recreation, relaxation and therapeutic effects, such as hydrotherapy. Indeed, it is estimated that more than 100,000 hot tubs are currently in use in the UK in both private and public settings. The UK is also ranked in the top 10 in the world hot tub market (BISHTA, 2015). The increase in hot tub ownership, coupled with the demand for eco-friendly products as well as awareness of the risks of incorrect maintenance, has led to an increased consumer demand for effective products against biofilms.

A biofilm can be described as a complex assemblage of microbial cells irreversibly adhering to a surface and encased in a self-produced matrix of extracellular polymeric substances, which can also contain non-cellular materials, such as corrosion degradation particles (e.g., iron oxide), and promotes bacterial survival and proliferation (Donlan, 2002 (Flemming et al., 2016).

A vast variety of microorganisms have been found in recreational waters, including spas, hot tubs and pools. Some of the disease-causing pathogens detected in recreational waters include *Campylobacter jejuni*, *Shigella sonnei*, *Cryptosporidium* spp., *Giardia intestinalis, Escherichia coli* and *Pseudomonas aeruginosa,* as well as bacteria of the *Legionella* genus (Fewtrell and Kay, 2015; Leoni et al., 2018). These (opportunistic) pathogens are most often the results of human fecal contamination, environmental factors such as sand and rainfall, and domestic or wild animals (Fewtrell and Kay, 2015). In addition to bacteria, a variety of bodily secretions such as vomit, mucus, faeces, saliva and skin flakes have been found in recreational waters (Thorolfsdottir and Marteinsson, 2013).

Traditionally, chlorine has been used as a disinfectant agent in spas and hot tubs because of its high oxidation potential. However, the high working temperature of the hot tub, in addition to water aeration caused by the jets, can lead to evaporation of the chemicals and induction of unpleasant effects on users. Additionally, chlorine can react with organic hot tub contaminants and lead to the formation of potentially toxic disinfection-by-products (DPBs) such as trihalomethanes, halomethanes (THMs), haloacetic acids and chloramines, and its biocidal abilities may be hindered by water hardness (Swanson and Fu, 2017; Valeriani, Margarucci and Spica, 2018). Bromine-based products, another popular option for microbial sanitation, suffer from sun degradation (Valeriani, Margarucci and Spica, 2018). Most importantly, however, chlorine disinfection has been found to increase antibiotic resistance

genes (Liu et al., 2018). Active oxygen, which describes oxidizing agents, both inorganic and organic, released from peroxide in aqueous solutions (such as hydrogen peroxide or benzoyl peroxide) (see section 1.14 for more details) is a promising alternative, with well-known antimicrobial effects which has been used for many decades in water decontamination (Choudhury et al., 2018). It has proven antimicrobial effects against *E. coli* and *P. aeruginosa,* two bacteria that can exhibit antibiotic resistance (Fontes et al., 2012). Therefore, an efficient hot tub sanitation treatment system using environmentally friendly alternatives combined with active oxygen provides an attractive option for hot tub owners.

1.2 Biofilm formation

Bacteria can be generally found in two states: planktonic, also known as free living, and sessile, adherent to a surface or as part of a biofilm (Garrett, Bhakoo and Zhang, 2008). A biofilm can be described as a "city of microbes", an organized microbial community encased in an extracellular polymeric substance matrix (EPS), which holds the biofilm together against a surface, and depending on the bacterial species, they may be made up for 10–25% cells and 75–90% EPS matrix (Costerton et al., 1987, Rasamiravaka et al., 2015). Once formed, the biofilm has an established architecture and allows bacteria to proliferate and survive. Biofilms can be found in various environments, such as medical devices, natural and artificial water systems and living tissue. They offer protection and are notorious for their resistance to antimicrobial agents, such as antibiotics and disinfectants, and being more tolerant against antimicrobial stress compared to planktonic cells (Donlan, 2002; Davies, 2003). In addition, through interactions between bacteria in combination with the properties of the extra cellular matrix, biofilms can develop new properties not normally observed in free-living cells (Flemming et al., 2016).

Biofilm formation is a multistep process which forms an endless cycle, generally consisting of five distinct stages (Fig.1)

1. Reversible adhesion on surface
2. Irreversible attachment
3. Colonization of surface / proliferation
4. Formation of a more structured phenotype / biofilm maturation

5. Bacterial dispersal and coverage of entire surface

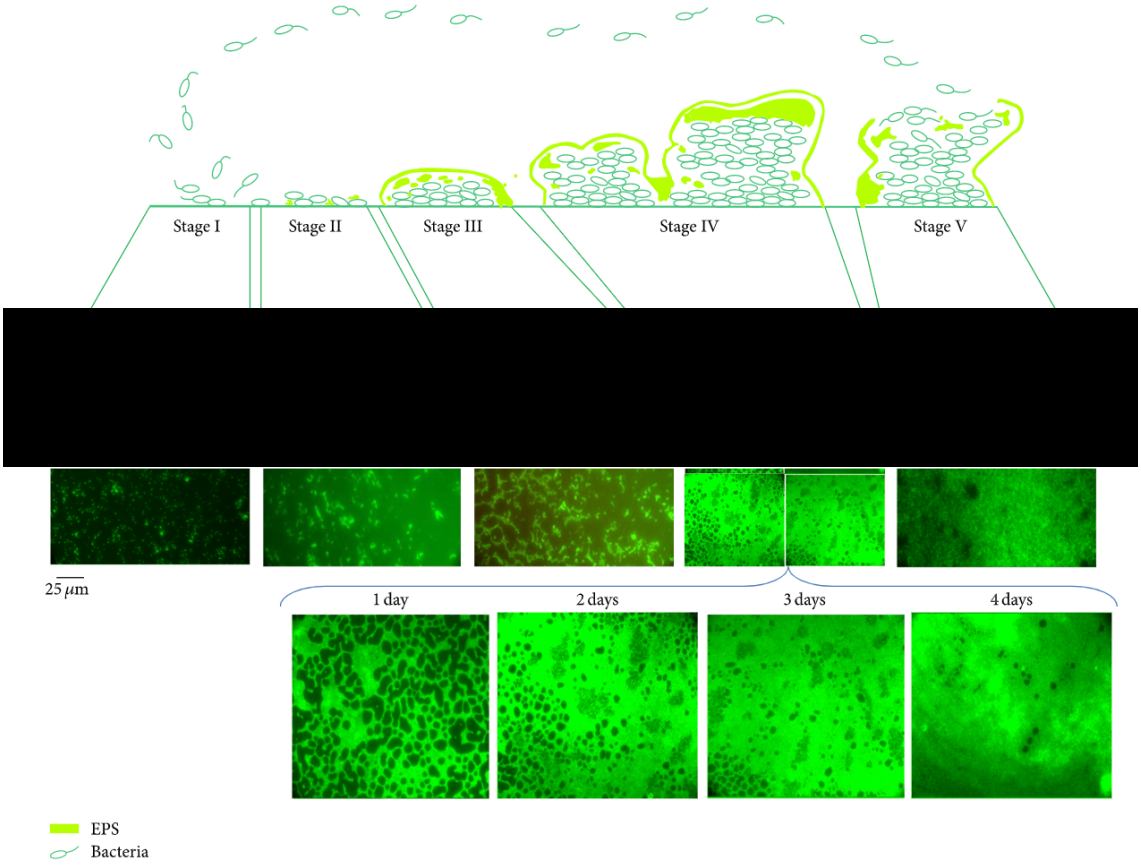


Figure 1.1: The formation of biofilm by P. aeruginosa in minimal medium on an abiotic surface: Top row of microscopy images: Stages I-IV. Bottom row: Stage IV over the course of 4 days. The fluorescent green color is caused by SYTO 9 staining which indicates live cells. (Rasamiravaka et al., 2015)

1.3 Conditioning film formation

The conditioning film - or layer, is the first building block of the biofilm, upon which the foundation of its development is provided. Starting with planktonic cells, the first step is bacterial adhesion to the surface, a complex step that can be influenced by many factors, such as cell surface hydrophobicity, presence of bacterial appendages such as fimbria and flagella and EPS production (Rasamiravaka et al., 2015). Of particular importance appears to be the hydrophobicity and texture of the surface, with planktonic bacteria readily adhering to more hydrophobic and rough surfaces. The reason behind this is the increased surface area in rougher surfaces and the diminishing shear forces (Donlan, 2002). Non-polar, hydrophobic materials such as plastic and metals are ideal for bacterial adhesion, thereby forming an obvious risk of biofilm development on hot tub surfaces and piping.

The conditioning layer alters the substratum favorably in order to provide an ideal foundation for biofilm development, especially in the aquatic environment (Bar-Zeev et al., 2012). In the initial stages of biofilm formation, which may last anywhere between a few hours and a few days, organic polymers dissolved in the overlying water and colloids start adhering to the surface, beginning the formation of the conditioning film, which bacteria will then utilize to adhere to the surface (Bar-Zeev et al., 2012). This layer is composed of large variety of molecules, such as polysaccharides, proteins, lipids, and humic and nucleic acids, and surface charge, potential and tension can all be altered through interactions with the layer and surface, in order to negate repulsive forces and lead to irreversible adhesion (Donlan, 2002; Bar-Zeev et al., 2012). One way this can happen is thanks to microbial appendages called fimbriae, which contain non-polar sites, which are present in both *P. aeruginosa* and *E. coli* and can help overcome the initial electrostatic repulsion barrier between cell and surface (Donlan, 2002; Connell et al., 1996; Ruer et al., 2007). Additionally, increased water temperature and nutrients, both common in the hot tub environment, lead to increased bacterial attachment to surface (Donlan, 2002).

1.4 Bacterial adhesion

Biofilm adhesion, which is part of this stage, is made up of two steps: Primary and secondary adhesion.

During primary or reversible adhesion which can also be described as “docking”, the cells are brought in close proximity with the surface, either randomly by physical forces such as water propulsion, or directed movement, such as chemotaxis, facilitated by microbial moving apparatus such as flagella movement (Dunne, 2002). The net sum of attractive or repulsive forces determines adhesion (Garrett, Bhakoo and Zhang, 2008). Such forces include van Der Waals forces, steric interactions and electrostatic (double layer) formation and temperature (Dunne, 2002).

During secondary or irreversible adhesion, which can also be described as “locking”, a fraction of the cells during primary adhesion are immobilized and irreversibly adhere to the surface (Garrett, Bhakoo and Zhang, 2008). As the physical bacterial appendages (flagella, fimbria and

pili) come in touch with the layer, the bacteria begin to produce exopolysaccharides and extracellular proteins that bind with surface materials and their appendages (Figure 2). Additionally, during this stage the bacteria adhere to each other or other organisms bound to the surface, leading to the formation of aggregates. Interestingly, the presence of one bacterial species can promote the adhesion of another, and such is the case with *P. aeruginosa* and *E. coli* where the former species encourages adhesion and biofilm formation of the latter (Dunne, 2002, Culotti and Packman, 2014), ultimately resulting in mixed species biofilms.

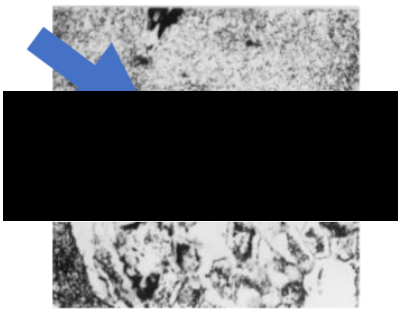


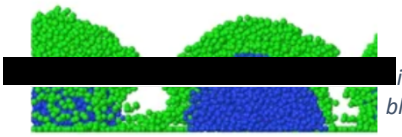
Figure 1.2: Bacterial microcolonies entrapped in a matrix of exopolysaccharide (Dunne, 2002)

1.5 Bacterial growth and maturation after adhesion

Following irreversible attachment, the bacteria begin to grow (binary division) and die on the surface. The growth of the bacteria is determined by many factors such as available nutrients, perfusion of dissolved gas and nutrients in the biofilm, bacterial waste removal, pH and oxygen perfusion (Dunne, 2002; Salgar-Chaparro et al., 2020). In particular, nutrient depletion has been found to play an important role in determining biofilm biomass and shape, with exposure to limited nutrients leading to thinner biofilm formation (Salgar-Chaparro et al., 2020). As bacteria divide, new cells spread outward and upward from the attachment point to form cell clusters (Hall-Stoodley and Stoodley, 2002). This results in the developing biofilm to adopt a mushroom-like structure (Fig. 3), which is thought to also assist with the spread of nutrients to cells deep into the biofilm (Garrett, Bhakoo and Zhang, 2008). In *P. aeruginosa*, the mushroom-like structure is caused by competition for nutrients amongst bacteria and is facilitated by rhamnolipids, whose production is upregulated during biofilm formation (Rasamiravaka et al., 2015; Ghanbari et al., 2016). Rhamnolipids are classified as biosurfactants, and are predominantly produced by Pseudomonas aeruginosa, and are

classified as mono and di-rhamnolipids (Sekhon Randhawa and Rahman, 2014). They play an important role as anti-adhesive molecules, promoting biofilm dispersion/disruption, and in the mushroom-like structure formation stage, they facilitate the formation of a motile subpopulation which can migrate up the stalks and form mushroom caps (Nickzad and Deziel, 2014).

Additionally, a study that looked into the mushroom-like structures formed by hydrodynamic *P. aeruginosa* showed that these structures are composed by both motile and immotile cells, and motile cells tend to "climb" the structure and settle on top of the immotile cells, leading to the "cap" formation. Interestingly, the nutrient level was noted to influence the structure; in low nutrient environments the stalks had high density as the cells were forced to migrate to the top (Ghanbari et al., 2016).



igure 1.3: Mushroom structure: Motile cells are green, immotile are blue (Ghanbari et al., 2016)

Following an initial lag phase, a sharp increase in the population can be observed, also known as exponential growth phase and is dependent on the environment and its conditions. It is at this point when processes aimed at adhesion cease and biological processes take over. The main aim at this stage is adhesion between cells, which is assisted by the production of polysaccharide intercellular adhesion polymers (PIA) and presence of divalent cations, which can interact with exopolysaccharides and thus modify the structure and composition of biofilm, and interact to form stronger intercellular adhesion (Garrett, Bhakoo and Zhang, 2008; (Steiger et al., 2020). In addition, extracellular DNA, which is produced after either active secretion or cell lysis, also plays an important role in biofilm formation. In particular, its adhesion and extension from the cell surface has been found to aid in bacterial adhesion at this stage, by providing structural stability of aggregates in the initial stages of biofilm development (Okshevsky and Meyer, 2015). In particular, it has been demonstrated that eDNA can bind to Type IV pili in *P. aeruginosa*, which forms a mesh in the biofilms and assists aggregation (Okshevsky and Meyer, 2015). Another factor aiding in cell-to-cell adhesion are extracellular structural biofilm proteins which make up the matrix (Flemming et al., 2016). The extracellular matrix, made up of self-producing polymers, also helps bacteria attach to each other, and is made up of exopolysaccharides, such as Pel and Psl which will be described

in more detail below, proteinaceous components such as functional amyloids and eDNA (Steinberg and Kolodkin-Gal, 2015)

Additionally, there are important gene expression changes associated with biofilm maturation post adhesion, such as upregulation of factors which promote and support attachment, especially of the extracellular matrix.

In *E. coli,* which has been extensively studied*,* cellulose and curli, both components of the *E. coli* biofilm, show formation at this stage (Kostakioti, Hadjifrangiskou and Hultgren, 2013). Curli, being important amyloid fibers of the extracellular matrix, are important to *E. coli* surface adhesion, cell aggregation and biofilm formation (Barnhart and Chapman, 2006). Other studies have observed polyglucosamine and colonic acid contribution to biofilm architecture (Kostakioti, Hadjifrangiskou and Hultgren, 2013). Additionally, lipopolysaccharides (LPS O antigen), capsules (polysaccharide K antigen) and ß-1,6- *N*-acetyl-D-glucosamine polymer (PGA) have been shown to be important components of the *E. coli* biofilm. PGA assists biofilm formation by aiding cell-to-cell adhesion and surface attachment, and also acts as an adhesin, stabilizing the *E. coli* biofilm. Colonic acid, another component of the *E. coli* biofilm, is capable of forming a capsule around the cells, offering protection (Sharma et al., 2016).

P. aeruginosa has also been studied extensively (Fig. 4). At least three extracellular polysaccharides have been observed: alginate, Pel and Psl, which are determinants of biofilm structure. Alginate is a linear unbranched polymer made up of D-mannuronic and L-guluronic acid and contributes to biofilm stability and nutrient retention (Rasamiravaka et al., 2015). PSL is a repeating pentasaccharide consisting of D-mannose, L-rhamnose, and D-glucose and has been shown to benefit attached cells and bacteria that cannot produce PSL, and provide tolerance against antibiotics (Irie et al., 2016). The positively charged Pel polysaccharide is mainly a glucose-rich polymer made up of partially acetylated 1→4 glycosidic linkages of N-acetylgalactosamine and N-acetylglucosamine, whose production is mediated by the pelA-G operon (Colvin et al., 2011; Jennings et al., 2015). Additionally, eDNA (extracellular DNA) is an important component of *P. aeruginosa* biofilms, by aiding cell-to-cell adhesion, biofilm expansion and early biofilm development (Rasamiravaka et al., 2015).

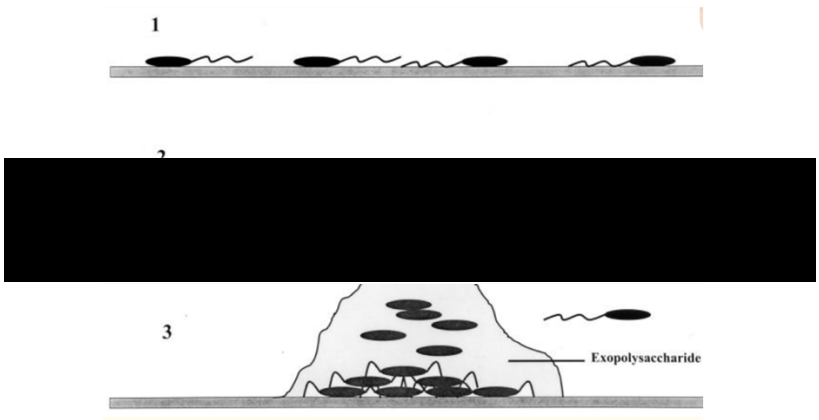


Figure 1.4: Graphic representation of biofilm formation by P. aeruginosa. Step 1: primary adhesion. Step 2: aggregation. Step 3: Exopolysaccharide production (Dunne, 2002).

1.6 Quorum Sensing

Once the cell concentration in the biofilm reaches a high enough number, a series of cell signaling processes, collectively named quorum sensing (QS) take place (Garrett, Bhakoo and Zhang, 2008). QS can be described as cell-to-cell communication employed by bacteria to control their population density by the production and reception of diffusible signaling molecules, which subsequently affect virulence, motility and biofilm formation (Rasamiravaka et al., 2015). Quorum sensing is especially important for virulence, as it has been observed that bacteria need to reach a certain population density in order to start expressing pathogenicity, affect the host and initiate disease (Li and Tian, 2012). *P. aeruginosa*, in particular, exploits two QS systems, *las* and *rhl (*Rasamiravaka et al., 2015). These two systems lead to the production of enzymes synthases LasI and Rhl II and the transcription factors LasR and RhlR of autoinducing signaling molecules. The *las* system has been shown to aid biofilm formation and maturation, and the *rhl* system has been linked to Pel and rhamnolipid synthesis. Additionally, there is a third*, Pseudomonas*-exclusive QS system, the PQS system, which is based on quinolone signals and is associated with eDNA release (Davies, 1998; Rasamiravaka et al., 2015). QS signals consist of acyl-homoserine lactones (AHLs), autoinducing peptides (AIPs) and autoinducer-2 (AI-2), which are key players in bacterial pathogenesis (Jiang et al., 2019).

Quorum sensing is also of interest as a therapeutic target and anti-biofilm factor. In particular, anti-QS factors are a novel approach and are being examined as an alternative to antibiotics, by inactivating QS receptors, inhibiting and degrading QS signals, and engineering QS-blocking antibodies that induce bacterial cell death (Jiang et al., 2019).

1.7 Final stages of biofilm maturation and dispersion

As the biofilm matures, bacterial dispersal and colonization of other sites becomes an option. Dispersal can happen both passively, e.g., by shear stress, but also actively by choosing to pursue a planktonic state. Bacteria have evolved ways to judge whether dispersal should occur or not, which is based on a plethora of factors, such as available nutrients, oxygen fluctuations and toxic product concentrations. Signaling is important for the change from adhesion to dispersion, and one signal that appears particularly important is the cyclic-dimeric-GMP (c-di-GMP signal), associated with changes in motility in both *P. aeruginosa* and *E. coli* (Kostakioti, Hadjifrangiskou and Hultgren, 2013). Additionally, the process is accompanied by the production of enzymes which help with EPS degradation, leading to the release of bacteria and colonization of surfaces elsewhere. One such enzymes is alginate lyase, produced by *P. aeruginosa*, and N-acetyl-heparosan lyase, produced by *E. coli*. At the same time, genes encoding for flagella proteins are upregulated, equipping the bacteria with the necessary apparatus for motility and genes encoding for porins become downregulated (Garrett, Bhakoo and Zhang, 2008).

1.8 Biofilm adaptation in static and dynamic environments

Hot tubs are often equipped with water jets, which can be turned on and create a dynamic enviroment, which can cause water projection and generation of shear stress which assists the spread of bacteria. Hydrodynamic forces are a key regulator of bacterial adhesion to substratum: on one hand, low shear stress can limit bacterial detachment and assist with cell adhesion, and, on the other hand, high shear stress assists with mixing and bacterial transport across the environment (Saur et al., 2017).

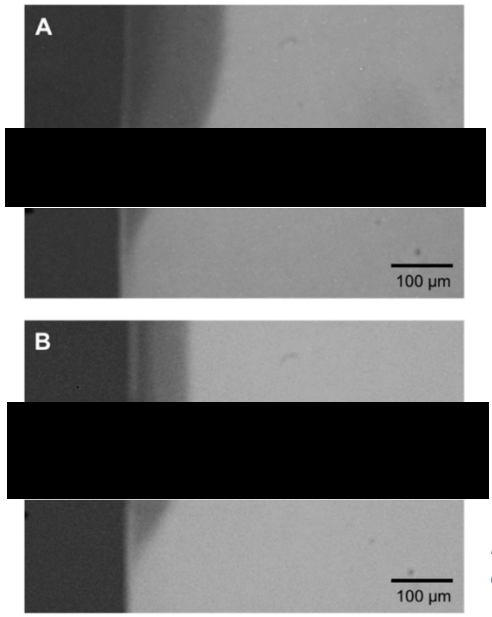


Figure 1.3: Comparison of biofilm formation on dentin on A: static and B: dynamic conditions (Santos et al., 2019)

Studies have shown that the jets lead to generation of vibrations and pressure and lead to biofilm consolidation, or in other words formation of a higher density biofilm over time (Figure 1.5). It has also been observed that water flow generates forces that "squeeze" the water out of the biofilm, and together with turbulent fluid flow over the biofilm lead to consolidation, especially at the bottom layers of the biofilm which undergo consolidation for the longest period of time, leading to them having the highest densities (Laspidou and Rittmann, 2004). Another study pointed out that hydrodynamic forces affect biofilm formation by controlling oxygen availability (Thomen et al., 2017). Thus, the hydrodynamic forces here lead to the development of a stronger, but thinner biofilm (Liu and Tay, 2002).

1.9 Phenazines and electron transfer

Phenazines are a group of natural products produced by a plethora of Gram-positive and Gram-negative bacteria, including *P. aeruginosa* (Guttenberger et al., 2017). Although there are many phenazines, the most important ones in *Pseudomonas* are phenazine carboxylate (PCA), phenazine carboxamide (PCN), and pyocyanin (PYO) (Saunders et al., 2020). They are colored compounds and pyocyanin, one of the most studied phenazine, is responsible for giving *Pseudomonas* its distinctive blue green color (Figure 6) (Price-Whelan et al., 2007).

These pigments are also redox-active, and have been shown to influence gene expression, metabolic flux, and redox balancing in their hosts (Price-Whelan et al., 2007).

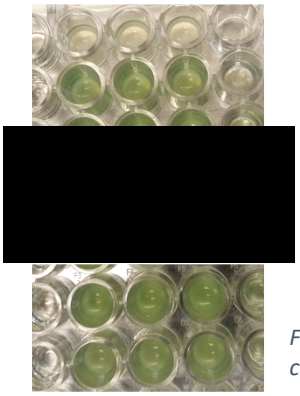


Figure 1.4: The characteristic blue-green color exhibited by P. aeruginosa cultures (Karagianni.,2020)

Most importantly, however, phenazines are responsible for promoting antibiotic resistance in *P. aeruginosa* biofilms. Studies have shown that biofilms from phenazine-null mutants treated with ciprofloxacin, a clinically relevant antibiotic, showed higher antibiotic susceptibility compared to wild type, phenazine-producing *P. aeruginosa* biofilms (Schiessl et al., 2019). The study also found a link between phenazines and electron transfer, suggesting that antibiotic resistance may be linked to respiration.

Extracellular electron transfer (EET) describes the process where electron acceptors or donors are accessed by nearby cells. During biofilm development, a concentration gradient of substrates such as oxygen is formed, as it is consumed by cells in the outer border of the biofilm faster than it can be diffused in the interior of the structure. In order to ensure oxygen-dependent electron acceptors can be accessed in the interior of the biofilm, mechanisms such as EET take place within the matrix (Saunders et al., 2020). A recent study established the role pyocyanin plays in an efficient redox cycle and pinpointed to the role of extracellular DNA in the facilitation of electron transfer, highlighting it as a key player in cellular respiration and the first example of a metabolically useful molecule bound by the extracellular matrix (Figure 7). In particular, it is hypothesized that oxidized pyocyanin is retained in the oxic region of the oxygen gradient, and reduced PCN and PCA are found in the anoxic region, where they can diffuse outwards towards the oxic region. There, they reduce the oxidized PYO, which reacts with oxygen leading to oxidation, and the re-oxidized PYO reacts with eDNA, which re-oxidizes PCN and PCA and can allow diffusion towards the anoxic region (Saunders et al., 2020)

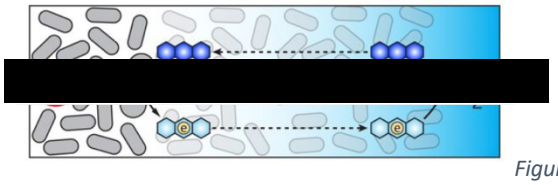


Figure 1. 5: The phenazine redox cycle in the biofilm. Cells are represented as grey shapes, phenazines as blue structures, electrons as circles and the oxygen gradient in the biofilm as a blue gradient (Saunders et al.,2020).

1.10 Cyclic di-GMP

c-di-GMP, which is synthesized by diguanylate cyclase (DGC), is well known for its role as an intracellular messenger responsible for the coordination of the "lifestyle transition" from lone, motile cells to sessile, biofilm forming cells and vice versa, and its activity has been demonstrated in a wide range of bacteria, including *E. coli* and *P. aeruginosa* (Valentini and Filloux, 2016; Cotter and Stibitz, 2007). C-di-GMP contributes to this transition by modulating gene expression profiles though interaction with transcriptional regulators, such as FleQ and PelD (Basu Roy and Sauer, 2014). Increased c-di-GMP levels are associated with sessile growth, while lower levels are associated with planktonic growth (Cole and Lee, 2015). For example, *P. aeruginosa* biofilms are estimated to contain on average 75–110 pmol of c-di-GMP per mg of total cell extract, whereas planktonic cells contain less than 30 pmol mg^{-1} (Basu Roy and Sauer, 2014). C-di-GMP has also been implicated in an emerging number of cellular functions, such as cell cycle regulation, differentiation, biofilm formation and dispersion, motility, virulence, and other properties (Figure 8) (Romling et al., 2013).

P. aeruginosa is a model organism to study the effects of c-di-GMP on cell motility. The messenger binds to many protein receptors, altering their function and leading to morphological changes. FleQ has also been shown to regulate the transcription of the *psl* and *pel* exopolysaccharide operons (Hickman and Harwood, 2008). In the absence of c-di-GMP, FleQ acts as a repressor, while in the presence of the messenger it acts as an activator (Hickman and Harwood, 2008). Pel synthesis requires activation of a seven-gene operon, *pelA-pelG*, all of which are essential for Pel-dependent biofilm formation (Colvin et al., 2013). A study has shown that the *pelA* promoter contains two FleQ binding sites on either side of its transcription start site, dedicated to activation and repression, which links to the observation that FleQ has a dual function (Baraquet et al., 2012). Additionally, high c-di-GMP

levels causes downregulation of FleQ-regulated flagellar genes which are associated with motility, such as *flhA* (Hickman and Harwood, 2008).

Moreover, high c-di-GMP levels, resulting in FleQ binding, also regulate the expression of the surface adhesin CdrA, a long, rod-shaped protein containing a beta helix structural motif (Borlee et al., 2010). Studies have shown that CdrA is important for the localization and stabilization of Psl, thus contributing to biofilm integrity and stability (Borlee et al., 2010). Additionally, c-di-GMP binds to PelD, a membrane bound c-di-GMP specific receptor. Studies have shown that c-di-GMP binding to PelD is essential for *Pel* polysaccharide production, further highlighting its importance to biofilm integrity (Whitney et al., 2012).

The messenger is also important in type IV pilus-mediated twitching motility, which is associated with biofilm formation and adherence (Kazmierczak et al., 2006). By binding with high affinity to FimX, a protein with a regulatory role in surface assembly of pili, it becomes localized to the leading pole of moving bacteria, where type IV pili assembly and retraction are taking place. Studies have shown that bacteria deficient in FimX exhibit impaired motility and microcolony formation (Jain et al., 2012). Interestingly, FimX becomes largely bound by c-di-GMP when P. *aeruginosa* transitions from liquid to surface-associated growth, as studies have shown a significant increase of intercellular c-di-GMP (Jain et al., 2017). Therefore, FimX may act to “license” pilus assembly at lower c-di-GMP concentrations typically exhibited following surface attachment, leading to directionally persistent movement and surface colonization (Jain et al., 2012).

Together, all these c-di-GMP receptor proteins act together to orchestrate the transition of planktonic bacteria to biofilms, with elevated c-di-GMP levels leading the process.

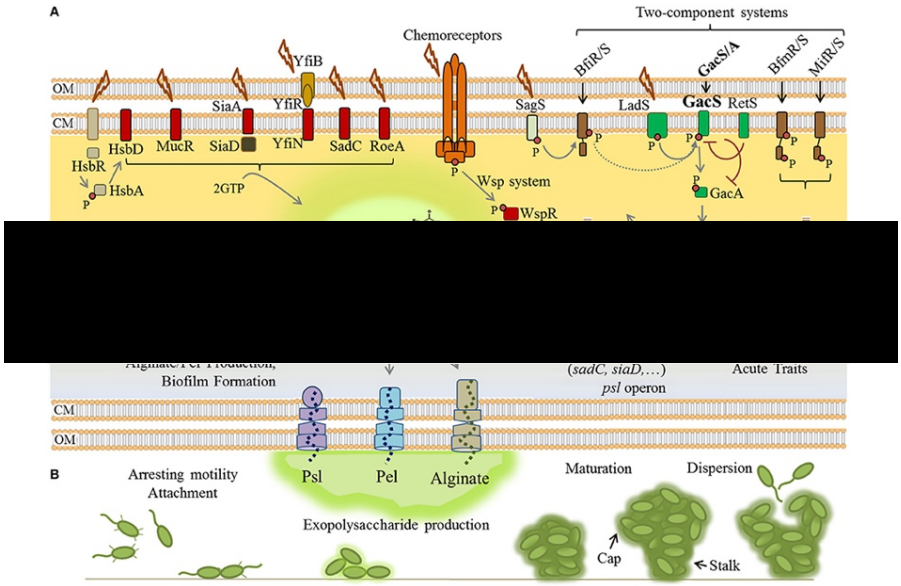


Figure 1.6: Regulatory networks underlying P. aeruginosa biofilm formation, showing the importance of c-di-GMP (Moradali, Ghods and Rehm, 2017)

However, a very important property of c-di-GMP is its ability to regulate antibiotic resistance in biofilms. It is well known that bacteria in biofilm show higher antibiotic resistance compared to planktonic cells (Nickel et al., 1985). As described above, the messenger plays an important role in biofilm stability, therefore directly influencing antibiotic resistance. A number of experiments have however hinted that c-di-GMP may itself influence resistance to antibiotics regardless of biofilm formation. For example, an experiment utilizing a *P. aeruginosa pel* mutant strain with high c-di-GMP levels showed higher fitness when compared with the same strain with low c-di-GMP levels, even in the planktonic stage (Nicastro et al., 2014). Another study, which focused on the genomics of adaptation of *P. aeruginosa*, showed that adaptive resistance to fluoroquinolones was due to multiple mutations in the known-resistance genes including the *gyrA, gyrB, nfxB*, and *orfN* which were associated with mutations in the genes involved in cyclic di-GMP signaling (Wong et al., 2012). In particular, *nfxB* encodes a negative regulator of the *mexCD-oprJ* genes, responsible for a multidrug resistance efflux pump involved in the extrusion of toxic substrates (Monti et al., 2013). This is of particular interest, as pyocyanin, whose properties were described previously and c-di-GMP both play key roles in regulating biofilm formation and multi-drug efflux pump expression in *Pseudomonas aeruginosa* (Wang et al., 2018).

1.11 Strains for analysis

A vast variety of microorganisms have been found in hot tubs, including *Campylobacter jejuni*, *Shigella sonnei*, *Cryptosporidium* spp., *Giardia intestinalis, Escherichia coli* and *Pseudomonas aeruginosa,* as well as bacteria of the *Legionella* genus (Fewtrell and Kay, 2015; Leoni et al., 2018). These pathogens are most often the results of human fecal contamination, environmental factors such as sand and rainfall, and domestic or wild animals (Fewtrell and Kay, 2015). *E. coli* and *P. aeruginosa,* two biofilm-forming bacteria commonly found in hot tubs, have exhibited antibiotic resistance (Fontes et al., 2012). Therefore, it was decided that these two bacteria would be relevant organisms for assessing the sanitizing efficacy of the products.

- *Escherichia coli* (*E. coli*) K12

E. coli is a Gram-negative bacterium and an anaerobic inhabitant of the gastrointestinal flora, where it exists as part of a multispecies biofilm. Additionally, it is a highly versatile bacterium, capable of being a common medical device contaminant, a frequent cause of urogenital infections and a hot tub contaminant (Beloin, Roux and Ghigo, 2008). Being a well-studied, reliable and convenient microorganism regarding its growth, *E. coli* is considered a valuable training and experimental model (Beloin, Roux and Ghigo, 2008; Brousseau et al., 2013). Moreover, *E. coli* presence in hot tubs is an indicator of fecal contamination. It is commonly sensitive to disinfectants and its presence suggests problems with disinfectant use or cleaning regime (Brousseau et al., 2013). *E. coli* has an optimum growth temperature of 37^{o}C and is able to grow in the temperature range between 30 and 42 degrees, making the working temperature of a hot tub ideal for its proliferation (Doyle and Schoeni, 1984).

- *Pseudomonas aeruginosa* (*P. aeruginosa*) PAO1

P. aeruginosa is a Gram-negative opportunistic pathogen, capable of causing folliculitis, otitis and respiratory infections such as pneumonia, especially in cystic fibrosis patients and immunocompromised populations (Lutz and Lee, 2011; Crnich, Gordon and Andes, 2003). Studies have shown that the two most common routes for contracting *P. aeruginosa* infections are through skin exposure in hot tubs and inhalation of aerosols which are often present during hot tub use (Mena and Gerba, 2009). *P. aeruginosa* has very little nutritional

requirements and proliferates at a range of temperatures varying from 4–42 °C, making the operational and standby temperatures of the hot tub, 25-37 °C ideal. Additionally, it is a common hot tub contaminant, with studies showing that up to 21-62.5% of hot tubs being positive for *P. aeruginosa,* with a recent study isolating *P. aeruginosa* in 20.9% of hot tubs examined (Lutz and Lee, 2011; Kush and Hoadley, 1980 (Caskey et al., 2018). Interestingly, *P. aeruginosa* also appears to promote corrosion of stainless steel (Yuan et al., 2008). *P. aeruginosa* also exhibits resistance to disinfectants, which makes it an ideal model organism to study the sanitation efficacy of the products (Russell, 1999)

1.12 Enzymes

As the water conditioner provided by the sponsoring company states that it contains enzymes, it was decided to perform a brief literature search on enzymes and their industrial applications, plus any potential applications in biofilm prevention and removal. After consultation by the sponsoring company, the researcher was informed that the water conditioner contains an enzymatic mixture, and the relative proportions of the enzymes were 90% Lipase, 5% protease and 5% amylase.

Enzymes are the most well-known biological catalysts (also known as biocatalysts), capable of accelerating biochemical reactions in living beings. They are globular proteins that vary in size from less than 100 to more than 2.000 amino acids can be arranged in one or more polypeptide chains which are then folded and bent to form a specific three-dimensional structure, including the active site, a small area where the substrate binds and is converted into product molecules (Robinson, 2015). They are affected by pH and temperature and have characteristic optimum pH where the speed of the catalyzed reaction is at maximum, and above and below which the velocity decreases (Robinson, 2015).. Due to the efficiency, they are commonly used in industry, especially the food and detergent industry, therapeutics, reagents and as tools in genetics (Robinson, 2015).

- Proteases

Proteases are degradative enzymes specialized in protein degradation and modification and make up for up to 20% of the enzyme market worldwide (Razzaq et al., 2019). Commercial proteases are produced by microbes because of their high yield, cost-effectiveness and desired characteristics (Razzaq et al., 2019). Proteases can be classified into serine, aspartic, cysteine proteases and metalloproteases, and are mainly sourced from *Bacillus sp.* They are active within a narrow pH range (5 to 8) and although optimum temperature can vary between source organisms, it is usually at around 60°C (Rao et al., 1998).

- Lipases

Lipases are a class of enzymes responsible for the hydrolysis of long chain triglycerides to free fatty acids and glycerol and are extensively used in the detergent industry (Snellman, Sullivan and Colwell, 2002). Lipases act under mild conditions, are highly stable in the presence of organic solvents, have broad substrate specificity and show high regio- and stereo- specificity in catalysis (Snellman, Sullivan and Colwell, 2002). Additionally, they are biodegradable, leave no harmful residue and are safe for aquatic life, and increase detergent efficacy by reducing the temperature needed for efficient cleaning (Hasan, Shah and Hameed, 2006).

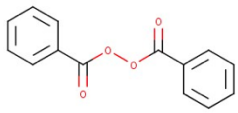
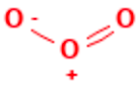
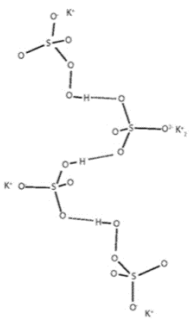
- Amylases

α-Amylases catalyze the hydrolysis of α-1,4-glycosidic linkages in starch, low molecular weight products, such maltose and maltotriose units, and in naturally abundant biopolymers composed of glucose units. They are usually sourced from either *Bacillus sp.* or *Aspergillus sp*. It exhibits optimum amylolytic activity at pH ranges between 7.5 and 11 and has different optimum temperatures depending on the source organism, ranging from 22°C-70°C. Additionally, in a study α-amylase showed reasonable stability in the presence of protease K, by retaining 71% of its activity at 75°C (Souza and Magalhães, 2010). Interestingly, commercial α-Amylases also show anti-biofilm potential in *Staphylococcus* aureus biofilms, which may be partly explained by the fact that amylose-like polysaccharides are present in the EPS (Bradford, 2011). Amylose-like polysaccharides (α 1-4 linked polymers) are also present in *P. aeruginosa*, and bacterial amylases have shown to be effective in *P. aeruginosa* biofilm prevention (Kalpana, Aarthy and Pandian, 2012).

1.13 Active Oxygen

Active oxygen is a blanket term referring to oxidizing agents, both inorganic and organic , with the term active oxygen referring to the active agent released from peroxide in aqueous solutions (Bloomfield, 2004). Active oxygen substances, such as hydrogen peroxide, are widely used in the medical field, in bleaching procedures, water treatment and purification of organic compounds (Bloomfield, 2004). Table 2 provides a summary of well-known active oxygen substances, their structures and most common uses.

Table 1.1: Examples of active oxygen substances and their uses (ECHA, 2021).

Name	Structure	Molecular formula	Uses
Hydrogen peroxide		H_2O_2	Biocide, cosmetics, pH regulator, water treatment
Benzoyl peroxide		$C_{14}H_{10}O_4$	Medication, water treatment, polymer production
Ozone		O_3	Biocide, water treatment, drinking water treatment
Pentapotassium bis(peroxymonosulphate) bis(sulphate)		$KHSO_5$	Water treatment, wastewater treatment, pollutant degradation

Active oxygen species have well known and well documented antimicrobial, fungicidal and virucidal effects, and have been widely used in water treatment, intraoperatively to prevent tissue infection by pathogens, and as a biocidal in dental operations such as root canal procedures. Their biocidal effects are based on their oxidative effects and formation of free radicals (Hunt and Mariñas, 1997).

One such species is ozone, a naturally occurring gaseous molecule of triatomic allotrope of oxygen, whose bactericidal effects are well known (Song et al., 2018). Oxidation reactions are caused by dissolved molecular ozone or free radical species created during the auto-decomposition of ozone (Hunt and Mariñas, 1997). Many studies have exhibited its anti-biofilm effects against *P. aeruginosa*, stating that as little as 30 seconds of exposure are enough to express bactericidal effects (Bialoszewski et al., 2011). Another study examined active oxygen's mode of action against *P. aeruginosa* and detected leakage of K^+, Mg^{2+} and ATP, and led to the conclusion that active oxygen inactivates bacterial cells by causing cytoplasm agglutination, protein denaturation and membrane permeabilization. The same study also found that exposure to ozone led to *P. aeruginosa* cells to exhibit sensitivity to sodium dodecyl sulfate, a widely used detergent, and subject to inactivation by proteinase K (Zhang et al., 2015). It has been shown that the lipoprotein and lipopolysaccharide of gram-negative bacteria are the first targets of ozonation (Kim,Yousef and Dave, 1999). Interestingly, a study showed that active oxygen may actually be more effective at killing gram-negative bacteria, including *E. coli* and *P. aeruginosa*, than gram-positive bacteria (Restaino et al., 1995). Ozone is also an interesting alternative compared to chlorine or bromine as it has a higher thermodynamic oxidation potential, produces less harmful disinfection by products and additionally has a better potential compared to chlorine as a coagulant aid; coagulation is a process where organic matter is removed during water treatment (Choudhury et al., 2018)

While performing literature search for the activity of Active Oxygen against bacteria, it was realized that the main active component in the tablets provided by the industrial sponsor was **pentapotassium peroxymonosulfate (PMS)**, in combination with potassium hydrogensulphate and dipotassium peroxodisulphate. PMS is the main ingredient in the widely used disinfectant Virkon (eco3spa user manual, 2019; ECHA, 2021). When diluted in

water, peroxymonosulfate produces hydroxyl radicals and sulfate-ion radicals, both of which possess potent antibacterial properties (Kennedy and Stock, 1960). Pentapotassium peroxymonosulfate is an oxidizing agent, and 1% solutions have shown excellent inactivation of viruses and bacteria, after 10 minutes of contact (Eleraky et al., 2002). A study on the bactericidal efficacy of Virkon against pathogens including *P. aeruginosa* and *E. coli* showed that after 5 minutes the product was active against both pathogens, achieving a 5-log reduction (Hernndez et al., 2000). In addition, another study on Virkon used at 1% concentration found it exhibits potent biocidal effects against both *P. aeruginosa* and *E. coli* (Gasparini et al., 1995). Another study found that Virkon at 0.003% showed an important inhibitory effect when added before an established population was formed (El-Naggar et al., 2001). Transmission electron micrographs (Fig. 1.9) showed the morphological changes after Virkon treatment, with the formation of spheroplasts visible at (Fig. 1.9 c) and cell lysis visible at (Fig. 1.9 d) (El-Naggar et al., 2001).

It is known that high temperature affects the activity of pentapotassium peroxymonosulfate. According to the safety data sheet of Virkon, high temperatures can lead to product disintegration and the formation of sulphur dioxide; therefore, Virkon treated cultures must not be autoclaved for this reason (Virkon S safety data sheet, 2021). However, despite an extensive literature search, information on the effect of pH on the activity of pentapotassium peroxymonosulfate was difficult to obtain and to the best of the researcher's knowledge, non-existent.

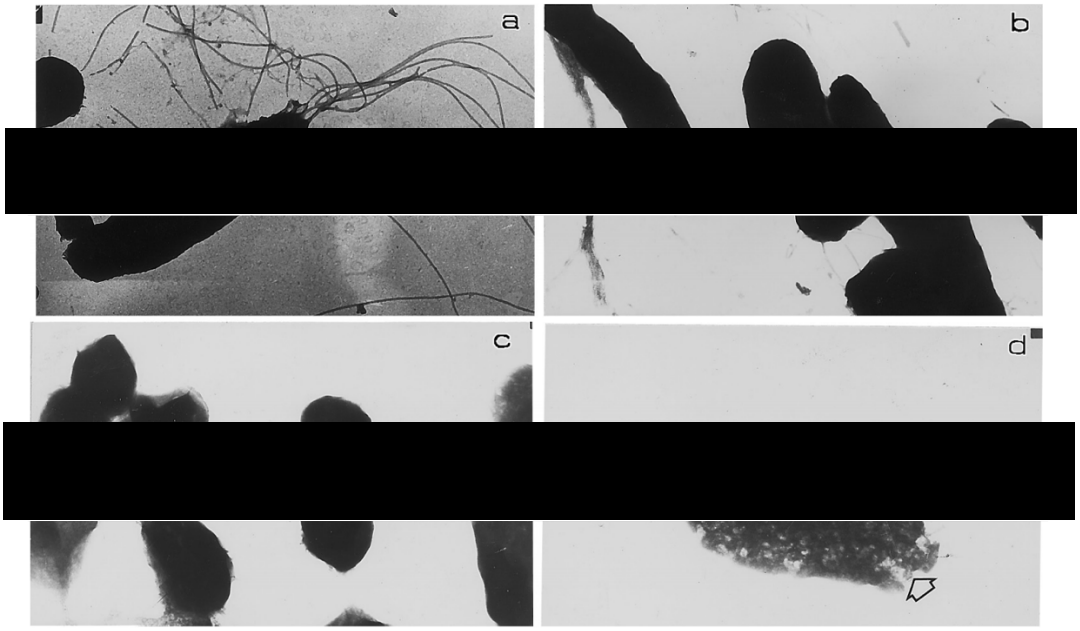


Figure 1.9: Transmission electron micrographs of E. coli after Virkon treatment. a (a) 0.03% Virkon-S for 60 min; (b) 0.03% for 360 min; (c) 0.125% for 60 min;(d) 0.25% for 15 min (El-Naggar et al., 2001).

1.14 Chlorine as a water treatment

Sodium dichloroisocyanurate (Figure 10), which is the main compound in the hot tub chlorine product (NaDCC) is a chlorinated cyanurate commonly used in swimming pools and hot tubs as a biocide and sanitizing agent against bacteria, viruses and parasites and has been approved as a treatment for drinking water (Clasen and Edmondson, 2006). Furthermore, it helps stabilize chlorine residuals by reducing sunlight-associated chlorine degradation. Chlorinated cyanurates provide cyanuric acid and free chlorine in the form of hypochlorous acid (HOCl), a weak acid, to the water (Jain et al., 2010; Wahman, 2018). Another benefit of dichloroisocyanurate is its stability, which may extend to years, and ease of use compared to other chlorine sources, such as liquid chlorine. Additionally, dichloroisocyanurate, in comparison to other forms of chlorine, produces a solution with a near-neutral pH, which is important as chlorine exhibits reduced sanitizing efficacy at low and high pH values (Figure 11) (Wahman, 2018). High and low pH values dissociate hypochloric acid, forming hypochloric ions (OCl-) (Mazzola et al., 2003). Hypochlorous acid is about 40 to 80 times more efficient in killing pathogens as compared to hypochlorite, and about 80 to 100 times more efficient in killing *E. coli* (Snoeyink and Jenkins, 1980). At the pH range of natural waters (pH 6-9), the amount of hypochlorous acid and hypochloric ions are very sensitive to pH fluctuations, and as their disinfection properties vary, pH monitoring and control can be critical in ensuring

sanitizing efficacy (Snoeyink and Jenkins, 1980). Interestingly, NaDCC gradually releases about 50% of its free chlorine when dissolved in water and even retains a "reserve" of chlorine, allowing for a 45-day lifespan of available chlorine at room temperature which gives it a significant advantage over chlorine which exhibits a lifespan of 24 hours (Parkinson et al., 1996; Clasen and Edmondson, 2006; Meireles et al., 2017).

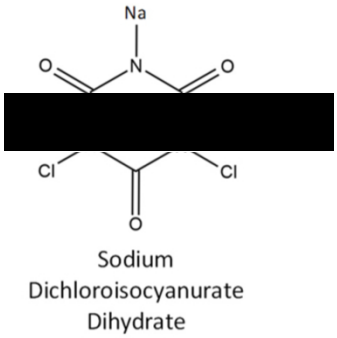


Figure 1.10: Sodium dichloroisocyanurate structure (Wahman, 2018)

Sodium dichloroisocyanurate has been used for a long time as a bactericidal agent against *E. coli*, *P. aeruginosa* amongst other pathogens (Bloomfield and Uso, 1985). Planktonic growth inhibition assays using *P. aeruginosa* in nutrient-rich media as a model organism have shown that NaDCC exposure for as little as 30mins is enough to significantly reduce growth. Exposure longer than 6hrs led to complete growth inhibition and proved its sterilizing abilities. Moreover, NaDCC concentrations of over 60% were found to inhibit *Pseudomonas* biofilm formation as well as inactivate active biofilms (Morgenthau et al., 2012).

Regarding *E. coli*, NaDCC exhibits the fastest antimicrobial action in comparison with other chlorine-based disinfectants, such as sodium hypochlorite (Meireles et al., 2017). NaDCC has been shown to effectively inactivate *E. coli* in concentrations as low as 750-1000 ppm and incubation times as short as three minutes (Long et al., 2020). Additionally, studies have shown its activity against *E. coli* biofilms, by showing a 2.8 $\log_{10}$ reduced biofilm viability (Chowdhury et al., 2019).

1.15 Eco3spa

The project involved collaboration with Eco3spa, a small company supplying hot tub chemicals and accessories throughout the UK and EU. Their range of products covers hot tub chemicals, filters, cleaning agents, covers and various accessories.

Eco3spa provides customers with the Eco3spa watercare kit, a novel 3-step hot tub treatment made from natural coconut, minerals and plant extracts and contains a hot tub cleaner, conditioner and active oxygen sanitiser tablets to last for 3 months of average hot tub use. According to the company, the enzymes in the product work synergistically with the eco3spa Water Sanitiser Tablets to increasing the effectiveness of the tablets and reduce the amount of sanitiser needed to maintain a safe hot tub. The company does not possess the scientific underpinning of efficacy and usage of the products in combination with each other and in different conditions such as varying temperature and pH. Work conducted during this study contributed towards the optimal performance of products, in terms of product concentrations, whilst also comparing product efficacy against the commonly used chlorine hot tub treatment, also supplied by the same company.

Table 1.2: Specific products analysed during the project

Name	Appearance	Purpose	Directions of use	Product Nr.
Eco3spa Hot tub cleaner	Blue liquid	Designed to remove biofilm from hot tub	Empty bottle in hot tub (1200 L)	1
Eco3spa Water Conditioner	Opaque liquid	Designed to condition the water, stabilizes pH	Use 300ml in hot tub (1200 L)	2
Eco3spa Active Oxygen tablets	Tablets	Design to prevent biofilm formation	Use two tablets in hot tub (1200 L)	3

1.16 Aims and objectives

Eco3spa has developed a 3-step biofilm removal and prevention kit which includes a biofilm remover hot tub cleaner, sanitizing tablets containing active oxygen, and an enzymatic water conditioner which is added to the water in combination with the active oxygen tablets. The recommended usage guidelines suggest using the hot tub cleaner first to remove any biofilm, then the water conditioner in conjunction with two active oxygen tablets to sanitize the hot tub and soften the water, and following that, use one tablet before each hot tub use.

As the recommended guidelines suggest the use of a variety of product combinations and concentrations, it was decided to assess the effects of the product combinations on biofilm removal and sanitizing efficacy. In addition, as the activity of chlorine is defined by a very narrow pH range, it was decided to compare the activity of active oxygen in a series of different pH and benchmark it against chlorine. The company was in agreement with the decisions.

Therefore, the aims set up with respect to Eco3spa's products were:

- To determine the optimal product concentrations, dosages, and combinations to prevent and remove biofilms

- To assess whether temperature influences active oxygen and other products components

- To examine whether various pH influence active oxygen, chlorine, and other products

- To determine the sanitizing efficacy of active oxygen and benchmark its activity against chlorine.

1.17 Experimental Strategy:

Although biofilms have been the focus of many studies over the past decades, little research has been performed in the prevention and removal of biofilms in the hot tub environment. The major aim of this work was to obtain an understanding of the activity of active oxygen tablets in various concentrations, under different pH and temperatures, and compare its activity to the competing product, chlorine. Biofilms in hot tubs have become a growing concern as research has shown they are not only present in a significant number of hot tubs and can affect vulnerable populations, but they are also capable of returning even following hot tub sanitation, as long as sanitation levels remain sub-optimal (Lutz and Lee, 2011). Moreover, there is concern over the development of resistance to chlorine, which is commonly used for hot tub sanitation (Mao et al., 2018). The Eco3Spa 3-step water watercare kit used to remove, sanitize and condition hot tub water will be tested to investigate its bactericidal properties and its activity will be compared to the competing chlorine product.

To achieve this aim, microtiter plate biofilm prevention and removal assays at two different temperatures (25 and 37°C) will be set up, using two different media (LB and M9). Selected strains of *E. coli* and *P. aeruginosa* will be diluted and exposed to each of the products or in product combinations in these assays. Benchmarking of active oxygen against chlorine by comparing optical density and crystal violet measurements will be performed (Chapter 3). Furthermore, planktonic growth following active oxygen treatment by itself or in combination with products will be monitored by measuring the optical density and biofilm formation measurements will be performed (Chapter 4). Moreover, live-dead staining and viability assays will provide more insights on the effect of active oxygen on cell morphology and viability (Chapter 5). Finally, protein assays will attempt to investigate the components of the water conditioner (Chapter 6) These experiments together generated accurate parameters in which the product is efficient of sanitizing the hot tub environment.

1.18 Covid-19 impact on research

Because of the effect of COVID-19 on lab access, and a 6-month absence from laboratories, the experimental time of this project was cut short. As a result, there were many limitations which affected this thesis.

Firstly, both LB and M9 media were supposed to be used in experiments. However, due to limited lab time and minimal medium M9 better representing the hot tub environment, it was decided to focus on this culture medium. The sponsoring company was in agreement to focus on this priority.

Additionally, many experiments were performed under time constraints, lab equipment limitations and under n=1 conditions. For example, upon return to laboratories in August for a 4-week period a rota system was in place that severely limited the available experimental time. Therefore, it was decided to prioritize only SOP experiments and combinations and only in M9 minimal media, and the sponsoring company was in agreement with this decision. Additionally, there was no time available to confirm or optimize any initial, non-SOP experiments. Moreover, many experiments and experimental procedures were performed for the first time, such as light and fluorescence microscopy and protein assays, which were performed under lab equipment limitations and under n=1 conditions.

Importantly, the project originally aimed to incorporate elements of chemical analysis which would have provided insights on the activity of active oxygen including the use of radical scavengers to measure the activity of Active Oxygen, and HACH assays to measure the activity and concentration of chlorine, which could potentially provide answers to some of the questions generated in this project. As the estimation of chlorine was made using test strips and their color could be subjective, there is a chance of inaccuracies in chlorine concentrations, and a more accurate chlorine measurement system could solve this. However, because of the fire on the Bay Campus which severely damaged the chemical engineering labs, it was impossible to arrange access to the necessary equipment.

Furthermore, because of lack of in-person courses, skills on graphing and statistical software were largely self-taught.

Chapter Two: Materials and Methods

2.1 Methods overview

This chapter describes the general materials and methods used for the experimental chapters.

2.2Bacterial Strains

The two bacterial strains used in the experiments were *Escherichia coli* K12 and *Pseudomonas aeruginosa* PA01 (Delaney, 2019).

Table 2.1: Strains used, genotypes and sources

Strain	Genotype	Source
Escherichia coli	K12	Frozen glycerol stock – Swansea ILS1
Pseudomonas aeruginosa	PAO1	Frozen glycerol stock – Swansea ILS1

2.2.1 Storage and treatment

All strains grown in LB media overnight were mixed with 500µl of 40% glycerol and stored as glycerol stocks. The glycerol stocks were stored at -80°C.

2.3 Media composition

Table 2.2: Media used and their composition (Sezonov et al., 2007; Varik et al., 2016)

Medium	**Composition**	**g/L**
LB-Luria - Bertani	Peptone	10
	Yeast extract	5
	NaCL	5
	Agar	12 (if required for plates)
M9 minimal media	Na2PO4	33
	KH2PO4	15

	NH4CL	5
	NaCL	2.5
	MgSO4	2
	Glucose	20
	CaCl2	0.1

2.3.1 Media Preparation

LB broth

To prepare nutrient rich broth, 20g of Luria-Bertani (LB) Lysogeny Broth powder was added to 1 liter of distilled water in a 1L glass bottle. This was then sterilized at 120°C for 20 minutes. To prepare agar, 20g of LB Lennox powder was added to 1 liter of distilled water in a media bottle. 15g of agar (Melford) was added and the bottle was autoclaved at 120°C for 20 minutes. Media was then poured into a series of 90mm sterile petri dishes and were left to solidify at room temperature within a preparation flow hood. The plates were stored in autoclavable plastic bags within a cold room at 4-8°C and were used within a week of preparation.

M9 minimal media

56.4g of Sigma-Aldrich M9 minimal salts (5x) was added to 1 liter of distilled water in a media bottle and dissolved. A 700ml of distilled water was then measured out in a fresh media bottle. 200ml of the sterilized M9 minimal salt solution was then added to the distilled water. This was then followed by: 2ml of $MgSO_4$, 20ml of 20% glucose solution and 100µl of 1M $CaCl_2$. The solution was then adjusted to 1L by adding more distilled water and autoclaved at 120°C for 20 minutes.

2.4 Buffers and dyes

Live-Dead stains

SYTO 9 was purchased for ThermoFisher. SYTO 9 is a green, fluorescent nuclear and chromosome stain with high affinity for DNA which can pass through intact cell membranes

and stain nucleic acids inside of them. Propidium iodide was purchased from ThermoFisher. It is a red, fluorescent DNA and RNA stain commonly used for bacterial viability assessment which only enters cells with ruptured or compromised cell membranes. Both dyes were dissolved in wells containing bacteria grown in M9 media, with or without active oxygen.

Phosphate buffered saline (PBS)

PBS is a sterile-filtered, liquid-based salt solution containing sodium phosphate and sodium chloride with a pH of 7.25. The components of PBS were Potassium phosphate (monobasic) 0.14g, Sodium chloride 9g and Trisodium phosphate 0.795g (per Liter).

Crystal violet solution (0.1%) w/v

1g of crystal violet powder was purchased from Sigma- Aldrich UK and dissolved in 499ml of distilled water in a sterile media bottle. The mixture was then shaken several times, wrapped in tin foil and stored away from light to prevent photodegradation.

Coomassie blue stain

1L of SimplyBlue Coomassie G-250 SafeStain was purchased from ThermoFisher and was used in accordance with the manufacturer's instructions.

2.5 Protein assays

2.5.1 Enzymes

According to the enzymatic product manufacturer, the three enzymes present in the water conditioner were lipase, protease and amylase, with a composition of 90% lipase, 5% protease and 5% amylase. Additionally, no trypsin or protease inhibitors were present in the product. After research on enzymes employed in similar applications, the enzymes purchased in aqueous solution from Sigma were:

Table 2.3: Enzymes ordered, source organism and protein amount

Enzyme type	Bacterial origin	Protein activity

Protease	*Bacillus amyloliquefaciens*	≥ 0.*8 U/g*
Lipase	*Aspergillus oryzae*	≥20,000 U/g
α-Amylase	*Aspergillus oryzae*	*≥800 FAU/g*

2.5.2 Pierce BCA assay kit

In order to determine the concentration of protein in the enzyme solutions and the water conditioner, a Pierce BCA (bicinchoninic acid) assay kit was purchased from ThermoFisher scientific. A series of BSA (bovine serum albumin) standard dilutions were prepared according to instructions and a standard curve was generated, which was used to quantify the amount of protein in the samples under investigation. The manufacturer's protocol of use can be found here: https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFSAssets%2FLSG%2Fmanuals%2FMAN0011430_Pierce_BCA_Protein_Asy_UG.pdf&title=VXNlciBHdWlkZTogUGllcmNlIEJDQSBQcm90ZWluIEFzc2F5IEtpdA==

2.5.3 Protein Precipitation procedures

In order to isolate any protein present in the water conditioner and remove any interfering substances, such as oils, two precipitation procedures were performed

Trichloroacetic acid reagent (TCA) precipitation procedure: 50 µl of undiluted water conditioner were pipetted in an Eppendorf together with 450 µl of ultrapure water, 100 µl of sodium deoxycholate and 100 µl of trichloroacetic reagent (72% (w/v) trichloroacetic acid in ultrapure water). The sample was allowed to incubate for 10 minutes at room temperature and was then centrifuged for 10 minutes at max speed. The supernatant was removed, and the pellet was resuspended in 5% SDS in 0.1 N NaOH.

Acetone precipitation procedure: 50 µl of undiluted water conditioner were pipetted in an Eppendorf and 200ul of cold acetone were added. The sample was vortexed and incubated for 30 minutes at -20^{o}C, after which it was centrifuged for 10 minutes at max speed. The supernatant was removed, and the pellet was resuspended in 50 µl of ultrapure water.

2.5.4 Protein gel electrophoresis

The protein gels used for the analysis of enzymes and the composition of the water conditioner were Novex NuPage 10% Bis-Tris Gels, purchased from ThermoFisher. The running buffer used was MOPS-SDS with a final pH of 7.7. The protein ladder used was "PageRuler™ Prestained Protein Ladder, 10 to 180 kDa".

The gel electrophoresis was performed according to the manufacturer's instructions. In brief, a small amount of each sample containing approximately 7.5 µg of protein (Table 2.3) was pipetted in a microcentrifuge tube together with 1µL Tris buffer, 4µL loading buffer and 2µL DTT (Table 2). Then, dH2O was added to each tube for a total volume of 25 µL, and the tubes were heated at 95^{o}C for 5 minutes.

Table 2.4: Enzymes used, their dilutions and amount of sample used

Sample	Amount of sample (approx. 7.5 µg protein)
Amylase (1:10 Dilution)	2µL
Protease (1:10 Dilution)	3µL
Lipase	1µL
Water conditioner	6µL

Samples (including 5 µL of Pre-stained Bis-Tris protein standards) were loaded into the gel and electrophoresis was conducted at constant voltage (140 V) at room temperature until the dye front reached the end of the gel after 80 minutes. The gel was then removed, washed and left to stain with Coomassie blue stain overnight. The gel was then destained with and preserved in 30% ethanol and once destained to a satisfactory degree it was photographed.

2.5.5 Lugol solution iodine amylase assay

To determine whether any active amylase was present in the water conditioner, Lugol solution was purchased from Sigma. Soluble starch was used as amylase substrate: 5g of soluble starch was added in 500ml of water and the mixture was boiled until transparent to form a 1% starch solution. In an Eppendorf, 1ml of starch and 1ml of the sample to be assessed were mixed and samples were removed at various time points, mixed with 100 µl of Lugol

solution in a microtiter plate and the color change was observed and compared to the positive control, which was the purchased amylase.

2.6 Products for biofilm prevention and removal

Table 2.5- Summary of the three products used in this study.

Name	Appearance	Purpose	Directions of use	Product Nr.
Eco3spa Hot tub cleaner	Blue liquid	Designed to remove biofilm from hot tub	Empty bottle in hot tub (1200 L)	1
Eco3spa Water Conditioner	Opaque liquid	Designed to condition the water, stabilizes pH	Use 300ml in hot tub (1200 L)	2
Eco3spa Active Oxygen tablets	Tablets	Design to prevent biofilm formation	Use two tablets in hot tub (1200 L)	3

2.6.1 Product 1 (Hot tub cleaner) dilutions

According to the manufacturer's instructions, all the contents of the bottle (500 ml) should be added to 1200 L (typical hot tub volume).

The dilution factor for 500 ml needed per 1,200,000 ml of water is 24000. The product was diluted prior to final use as 400 µl added to 480 ml water, creating a 12000 times dilution (2x working concentration). The final concentrations for use in microtiter plate wells (working concentration, total well volume 200 µl) were prepared by pipetting 100 µl of the intermediate solution into 100 µl of bacteria.

2.6.2 Active Oxygen tablets dilutions

According to manufacturer's instructions, two tablets should be added per 1200 L of water. The weight of two tablets is approximately 40g. The detailed preparation of active oxygen according to SOP is described in figure 2.1.

The dilution factor for 40 g needed per 1,200,000 ml of water is 30000. The product was diluted to reach the working dilution for first sanitation of a hot tub. As it was not possible to add to 0.01g in 300ml, 0.2 g was added to 300 ml water, creating a 1500 times dilution (20x working concentration). The final concentrations for use in microtiter plate wells (working concentration, total well volume 200 µl) were prepared by pipetting 100 µl of the intermediate solution into 100 µl of bacteria (see figure 2.1 for details).

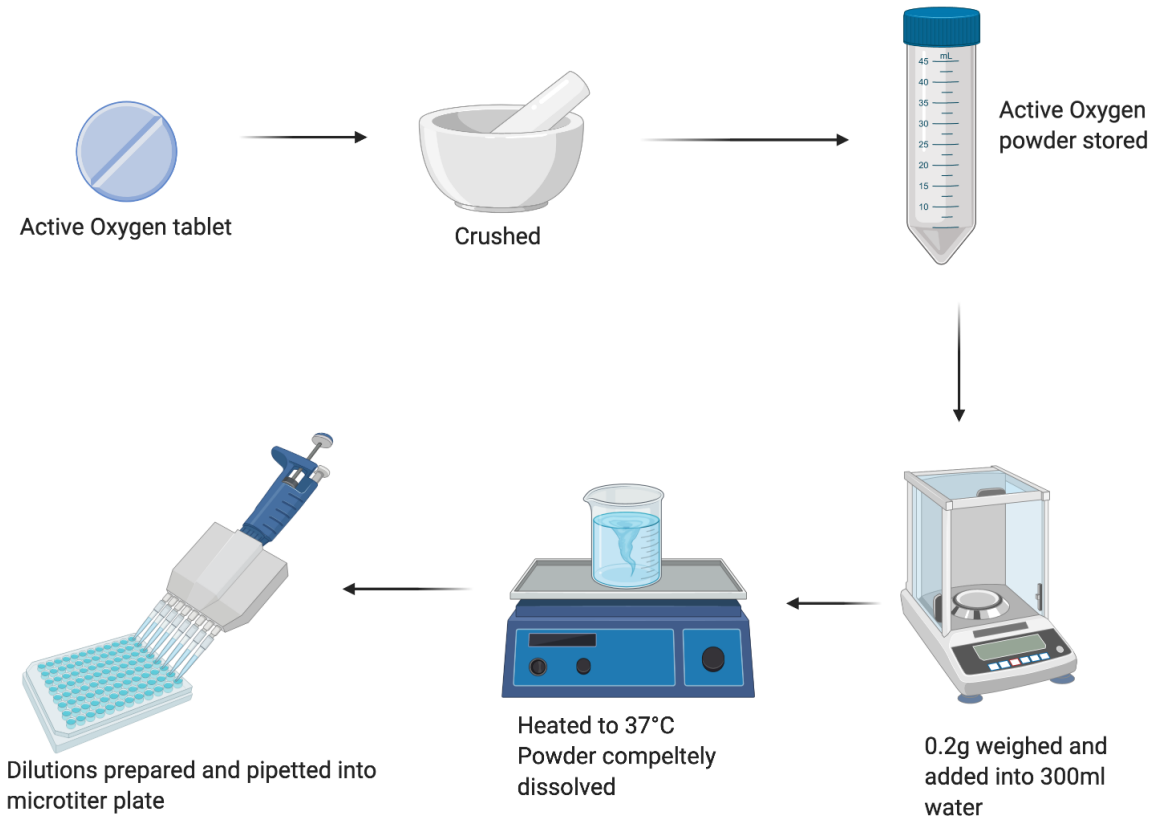


Figure 2.1: SOP preparation of Active Oxygen

From the 20x concentrated stock solution, a series of dilutions were prepared.

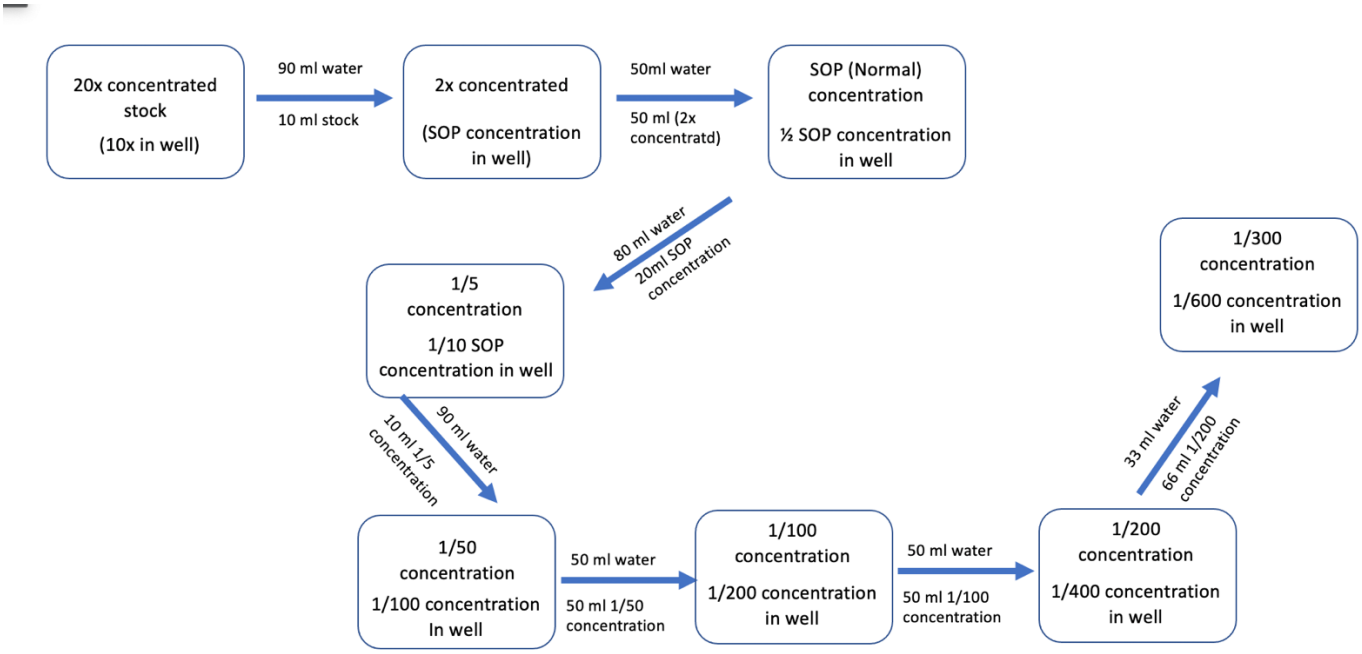


Figure 2.2: Illustration of the serial dilution methods used to make up the solutions of active oxygen

2.6.3 Water conditioner dilutions

According to the manufacturer's instructions, 300ml should be added to 1200 L (typical hot tub volume) every three months.

The dilution factor for 300ml needed per 1,200,000 ml of water is 40000. The product was diluted prior to final use as of 250 µl added to 500 ml water, creating a 20000 times dilution (2x working concentration). The final concentrations for use in microtiter plate wells (working concentration, total well volume 200 µl) were prepared by pipetting 100 µl of the intermediate solution into 100 µl of bacteria.

2.7 96 well-plates for static biofilm assays

The 96 well plates were purchased from Sigma-Aldrich UK and were kept in room temperature and in their original sterile packaging (Table 2.5)

Table 2.6: Specifications of 96-well plate used

Name	Costar 3599
Material	Polystyrene
Treatment	Tissue culture treated
Well volume	360µl
Well working volume	Up to 200µl
Plate geometry	Flat bottom

2.7.1 Static biofilm prevention assays

Using sterile tweezers in a flow hood, a sterile toothpick was dipped into a single colony on a streaked LB agar plate and suspended in 20ml of either LB or M9 media in a 20ml sterile universal tube for 16-18 hours at a shaking incubator at 37°C. When the optical density (OD) from the overnight culture was measured using a 1cm cuvette and had reached 1, a dilution of 1:100 was performed by pipetting 20µl of the overnight culture into 1.980µl of media in a 2ml tube. The diluted sample was then vortexed and 100µl was aliquoted into the wells of the Costar 96-well plate. Three replicates of control wells were also used, containing only media. Different plates were used for each bacterial strain.

100µl of the prepared active oxygen dilutions and biofilm remover dilutions were added to the wells. Then, the provided lid was used to cover the plates, and they were placed in two incubators at 37°C and 25°C for 24 hours under static conditions.

After 24hrs incubation, the planktonic growth of the cells was measured through optical density (OD) by using a BMG FluoStar plate reader at 595nm, with the path-length correction setting activated. After measurement, the liquid cultures in the plates were carefully emptied in a biohazard bag, washed once by pipetting 200µl of PBS into the wells, then emptied again into a biohazard bag. For plates containing *Pseudomonas*, a total of three washes of PBS were used. After that, the plates were allowed to air-dry partially inverted in the flow hood for 2 hours.

Once the drying step was complete, 200µl of 0.1% crystal violet solution was pipetted into the wells and the plates were incubated at room temperature for 15 minutes. The crystal

violet solution was then emptied in a biohazard bag and the stained plates were allowed to dry overnight. The following day, the crystal violet stain was re-solubilized by using 200µl of 30% acetic acid solution and incubating at room temperature for 15 minutes. 200µl of resolubilized dye was then transferred into a fresh 96-well plate and the OD was measured using a BMG plate reader at 595nm.

The OD was then measured at a wavelength of 595nm, which shows the absolute biofilm formation. Overall, the terms used to describe biofilm and growth were defined as:

Planktonic growth is defined as the OD595 (in AU) of the liquid bacterial culture itself, measured in the titer plates

Absolute biofilm formation is measured by the crystal violet measurement at OD595 (in AU) following staining and destaining of the cells adhering to the microtiter plate surface

Specific biofilm formation is defined as normalised biofilm formation (no unit) by dividing the absolute biofilm measurement of crystal violet at OD595 by planktonic growth measurement at OD595

2.7.2 Comparisons of Inner and Outer wells of 96 well plate

To determine whether there was any difference between the OD readings taken on inner wells of 96 well plates and the outermost wells due to potential edge effects, an average of 10 OD readings from wells in the inner most section of the plate was compared to an average OD reading from the outer most section of the plate. More details will be provided in the optimization chapter (Chapter 3).

2.7.3 Spectrophotometer

The spectrophotometer used to measure the OD of overnight cultures and their dilutions, as well as the OD of the standards of the Pierce BSA assay was a “DU 730 UV/Vis” spectrophotometer produced by Beckman Coulter, which utilized disposable cuvettes of 1ml max capacity and 1cm path length.

2.7.4 Microplate reader

The microplate reader used to measure the OD of 96 well plates throughout the project was a “FLUOstar Omega Microplate reader” produced by BMG biotech. Omega Software V5.70 was used for data interpretation, and the path length correction setting was applied throughout measurements.

2.7.5 Static biofilm removal assays

Using sterile tweezers in a flow hood, a sterile toothpick was dipped into a single colony on a streaked LB agar plate and suspended in 20ml of either LB or M9 media in a 50ml sterile Falcon tube for 16-18 hours at a shaking incubator at 37°C. When the OD (595 nm) from the overnight culture reached 1, a serial dilution of 1:100 was performed by pipetting 20µl of the overnight culture into 1.980µl of media in a 2ml tube. The diluted sample was then vortexed and 100µl was aliquoted into the wells of the Costar 96-well plate. Three replicates of control wells were also used, containing only media. Different plates were used for each bacterial strain.

The provided polystyrene lids were used to cover the plates, and they were placed in two incubators at 37°C and 25°C for 24 hours under static conditions.

After 24 hours, 50µl samples from each well were pipetted into a fresh 96-well plate and the planktonic growth was determined by measuring the OD using a plate reader set at 595nm. 100µl of the prepared active oxygen dilutions and biofilm remover dilutions were added to the inner wells of the plate. Then, the provided lid was used to cover the plates, and they were placed in two incubators at 37°C and 25°C for a further 24 hours under static conditions.

50 µl samples from each well were pipetted into a fresh 96-well plate and the OD was measured at 595nm. After measurement, the plates were emptied in a biohazard bag and 200µl of PBS was pipetted into the wells, then emptied into a biohazard bag. For plates containing *Pseudomonas*, three washes of PBS were used. After that, the plates were allowed to dry in the flow hood for 2 hours.

Once the drying step was complete, 200µl of 0.1% crystal violet solution was pipetted into the wells and the plates were incubated at room temperature for 15 minutes. The crystal violet solution was then emptied in a biohazard bag and the stained plates were allowed to

dry overnight. The following day, the crystal violet stain was re-solubilized by using 200µl of 30% acetic acid solution and incubating at room temperature for 15 minutes. The resolubilized dye was then transferred into a fresh 96-well plate and the OD was measured using a BMG plate reader set at 595nm.

2.8 Generation of a cell count vs OD vs CFU calibration curve

A sterile toothpick was dipped into a colony of either *P. aeruginosa* or *E. coli* on a streaked LB agar plate using forceps and suspended in 20ml of LB media in a 50ml sterile Falcon tube for 16-18 hours at a shaking incubator at 37°C. A series of dilutions were performed in microcentrifuge tubes resulting in a range of optical densities measured in cuvettes, ranging from 1.0 to 0.1. 20 µl Samples were taken from the tubes containing solutions with ODs of 0.1, 0.5 and 1 and were diluted and pipetted on a Helber Thoma counting chamber. The cover slip was placed on top, and the cells were counted with a 40x objective lens. 200 µl of each optical density preparation were pipetted to cover a vertical row of wells of a sterile 96-well plate, and using a multichannel pipette, 10-fold serial dilutions were prepared by pipetting 20 µl from the first column of wells into 180 µl of LB in the next column. The mixing function was used to thoroughly mix the dilutions in each column before moving on to the next. Following that, using the multichannel pipette, 10ul samples from each serial dilution were pipetted (N=1) onto an appropriately marked square LB plate which was then allowed to dry in the flow hood for 30 minutes before being incubated at 37 degrees for 16 hours. The colony forming units (CFUs) were counted at the dilutions where individual colonies could be identified and a calibration curve establishing the relationship of OD vs CFUs vs Cell counts for both *E. coli* and *P. aeruginosa* was generated. It is important to note that cuvettes were used for these measurements and pathlength correction was assumed of titer plate wells in further experiments.

2.9 Cell viability assay

To determine the number of viable cells after product addition a 10 µl sample from a biofilm prevention assay was diluted and plated on an LB agar plate. The plate was then incubated at 37^{o}C for 24 hrs. Colony forming units (CFU) were then determined by counting the number of colonies that had grown on the plate.

2.10 Fluorescence microscopy

For the visualization and quantification of planktonic live/dead cells, 200 µl samples from a biofilm prevention assay in M9 minimal media were pipetted in a new 96-well plate and 0.06 µl of SYTO 9 green, fluorescent nucleic acid stain, as well as 0.06 µl of Propidium iodide were directly added in the wells. Control (untreated) wells were used as a positive control and chlorine-treated wells (5ppm) were used as a negative control. The microscope used was a ZEISS Axioscope and the magnification used was 40x.

2.11 Statistical analysis of results

A range of statistical test were carried out to determine the statistical significance of the treatments on growth and biofilm removal and prevention. These tests include normality tests (Kolmogorov-Smirnov and Shapiro-Wilk), which led to the use of either parametric tests such as ANOVAs, or non-parametric multiple comparison tests, such as Kruskal-Wallis, Mann-Whitney U-test and Schreier-Ray-Hare (Ennos and Johnson, 2012). The software used to carry out the statistical analysis was GraphPad Prism version 8 and IBM SPSS version 26. P values that indicated no significant difference were $p>0.05$. In this thesis significant p-value scores are indicated by an asterix * indicates $p<0.05$, ** indicates $p<0.01$ and *** indicates $p<0.001$.

Chapter Three: Optimization, calibration and benchmarking

3.1 Introduction

In this chapter the objectives were to establish the experimental conditions that would allow optimal laboratory and high-throughput analysis of biofilm formed by each of the bacterial species: *E. coli* K12, and *P. aeruginosa* PAO1, which could be assumed to simulate conditions found in a hot tub. Experimental parameters and conditions tested included: media selection, incubation temperatures, incubation periods, washing/staining of biofilms, biofilm quantification, preparation of product dilutions and pH. The justification for the use of these conditions and methods is described below, supported by relevant published literature and observations made as part of the experimental process.

3.1.1 Media selection

LB media

Luria-Bertani (LB) media is commonly used for routine cell culture and maintenance because its nutrient-rich profile allows for quick bacterial growth and good growth yields. The media is rich in organic oligopeptides, allowing for access to a wide pool of free amino acids that support growth (Sezonov, Joseleau-Petit and D'Ari, 2007). Moreover, it has been shown that *P. aeruginosa* grows well on LB media (LaBauve and Wargo, 2012). Additionally, the nutrient-rich environment provided by LB media was considered similar to the nutrient-rich environment found in hot tub piping. Therefore, LB media is an ideal choice for assessing bacterial growth and biofilm formation for this project.

M9 minimal media

Glucose minimal medium (M9) is commonly used for bacterial culture, especially *E. coli*. It is made up of nitrogen sources and minimal salts, and a carbon source such as glucose (Rugbjerg, Feist and Sommer, 2018). It has been shown to support *P. aeruginosa* growth and biofilm formation, as well as *E. coli* growth and biofilm formation (Hammond et al., 2010; (Naves et al., 2008). Moreover, the minimal nutrient profile of the M9 medium simulates the

accessible water of the hot tub, providing an ideal medium choice for bacterial growth and biofilm development.

3.1.2 Incubation temperature

Two incubators set at 25 and 37 degrees Celsius were used in this project, in order to simulate the two operating temperatures found in a standard hot tub. In standby mode, the water is maintained at 25 degrees, while 37 degrees is the operational temperature of the hot tub (Hot tub manual, 2019). *E. coli* K12 has been shown to form biofilms to temperatures even at 23 degrees Celsius, and more interestingly, temperatures at this range have been shown to increase expression of genes associated with biofilm formation, leading to increased biofilm production compared to higher temperatures (White-Ziegler et al., 2008). *P. aeruginosa* has been observed to form biofilm in a range of temperatures ranging from 4 to 42 degrees Celsius and interestingly increased temperatures increase *P. aeruginosa* resistance to disinfectants (Lutz and Lee, 2011; Abdallah et al., 2015). Additionally, 37 degrees is the average body temperature, so studying biofilm formation at this temperature can provide interesting insights into biofilm and infection.

3.1.3 Incubation time

Both *E. coli* K12 and *P. aeruginosa* PA01 initial cultures were incubated overnight at 37 degrees for 16-18 hours, which is based on previous experiments which have used the same incubation time for both species (Singh et al., 2005). Once the overnight cultures had been pipetted into a 96 well plate, it would be then incubated for 24hrs at 37 degrees to allow biofilm to form. This is based on previous research which has indicated that the optimal *E. coli* biofilm formation takes place after 24 hours (Adamus-Białek et al., 2015). Similarly, previous research has shown that *P. aeruginosa* shows the optimal biofilm formation at 24hrs, which is also linked to increased antibiotic and disinfectant resistance (Cochran et al., 2000).

3.1.4 Phosphate Buffer Saline (PBS) washes

PBS is a salt solution with a neutral pH of 7.2, commonly used for variety of cell culture applications such as washing cells before dissociation, transporting cells or tissue, cell dilutions prior to counting and reagent preparation. In this project, PBS was used for the

washing step of planktonic bacterial cells before crystal violet staining so that levels of absolute and specific biofilm could be measured. According to previous research, PBS is effective for *E. coli* and *P. aeruginosa* planktonic cell washing and excess biofilm dye rinsing (Hung et al., 2013; Shen et al., 2012).

Additionally, after considering observations from initial experiments and previous research (Delaney, 2019), it was concluded that three PBS washes are necessary for sufficient removal of planktonic *P. aeruginosa* cells from the wells, which would otherwise lead to inaccurate measurements. Contrarily, only one PBS wash was sufficient for *E. coli* planktonic cell removal, anymore and the adherent biofilm would begin to detach (Delaney, 2019).

3.1.5 Crystal Violet solution

Crystal violet staining is an integral part of the microtiter plate biofilm assay, which is often considered the 'gold standard" for biofilm quantification (Haney et al., 2018). Crystal violet binds to negatively charged surfaces, such as cell membrane surfaces and polysaccharides in the biofilm matrix (Li et al., 2003). The suitable preparation includes a 0.1% solution of crystal violet wrapped in tin foil. Crystal violet has a long history of being used as an antiseptic and staining agent (Maley and Arbiser, 2013;). Moreover, it is widely used for *E. coli* and *P. aeruginosa* biofilm staining and quantification (Wakimoto et al., 2004; Knezevic and Petrovic, 2008). The staining was applied on well plates previously washed by PBS and according to published protocols, incubated at room temperature for 15 minutes before staining disposal (O'Toole, 2011).

3.1.6 Acetic acid solubilization

Following overnight incubation of the stained plates at room temperature, the dried crystal violet was re-solubilized by using a 30% acetic acid solution. After an incubation time of 15 minutes, the re-solubilized stain was pipetted in a fresh well plate and the optical density was measured at 595nm using a plate reader. Acetic acid has been used for re-solubilization of both *E. coli* and *P. aeruginosa* stained biofilms (O'Toole, 2011; Coffey and Anderson, 2014).

3.1.7 "Edge effect" in titer plate assays

Microtiter plate assays are considered the gold standard in biofilm detection and quantification (Haney et al., 2018). However, a common issue with the assay is the observed "edge effect", commonly affecting the periphery of the titer plate. The edge effect can occur largely due to two reasons. Firstly, water and liquid can evaporate more quickly from peripheral wells, therefore allowing planktonic cells to adhere to the walls, which ultimately leads to higher biofilm formation, giving an inaccurate reading. Secondly, the peripheral wells are better ventilated and could therefore allow more oxygen for bacterial proliferation. This effect is well documented, commonplace and difficult to avoid, with a large number of studies showing the outer wells of a 96-well plate showing increased optical density readings compared to the inner wells (Lundholt et al., 2003; Gordon et al, 2007; Falls et al., 2014).

In this project during the initial microtiter plate experiments, the edge effect was also observed. More specifically, the outermost rows of the 96-well plate were found to have abnormally high optical densities compared to the innermost wells. A number of methods have been described as solutions to the "edge effect", such as incubating the plate for one hour at room temperature before introducing the plate to the incubator (Lundholt et al., 2003). Another method of reducing the "edge effect" involves the replacement of the 36 outermost wells with water or growth medium in place of seeded wells, however a major drawback is the reduction of the usable wells. In fact, it has been estimated that there is a 38% reduction in the experimental output. Regardless, for the sake of obtaining replicable results in an appropriate time frame, it was decided to exclude the outermost wells and a comparison between the outermost and innermost wells will be described (Figure 3.1).

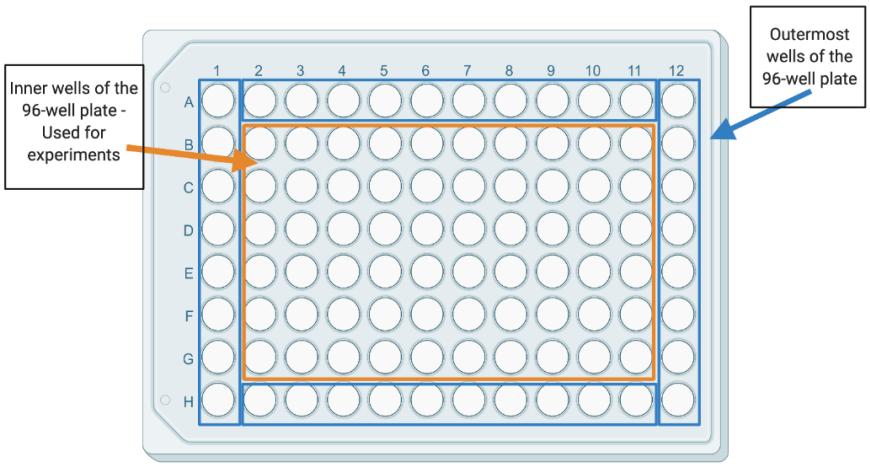


Fig. 3.1 - A typical view of a 96-well plate. Highlighted are the outermost (blue) and innermost (orange) wells of the plate, the latter were used for experimentation.

3.2 Results

3.2.1 Comparison of inner and outer wells

As the edge effect is often encountered in 96-well plate assays, it was considered important to determine whether this would be the case in this project, and if so, which wells would be affected and how significant the difference would be. An overnight culture in LB was diluted 1 in 100 and 100µl were pipetted in each well. Measurements took place after 24hrs. Results showed that there are significant differences between the inner and outer wells in both species (Fig. 3.2 and 3.3) with the exception of *P. aeruginosa* at 25°C where no significant differences were noted between inner and outer wells (Fig. 3.3).

In order to determine the effect of titer plate geometry on cell growth, its quantitation and the effect of temperature (if any), cell densities were compared in inner and outer-most wells. Figures 3.2 and 3.3 show the optical densities of *E. coli* and *P. aeruginosa* cultures in the outermost and innermost Co-star 3599 wells in nutrient-rich media (LB), after 24hrs

incubation at 37 and 25 degrees Celsius. The average OD was determined by taking an average of six replicate wells in the innermost and outermost wells of the plate.

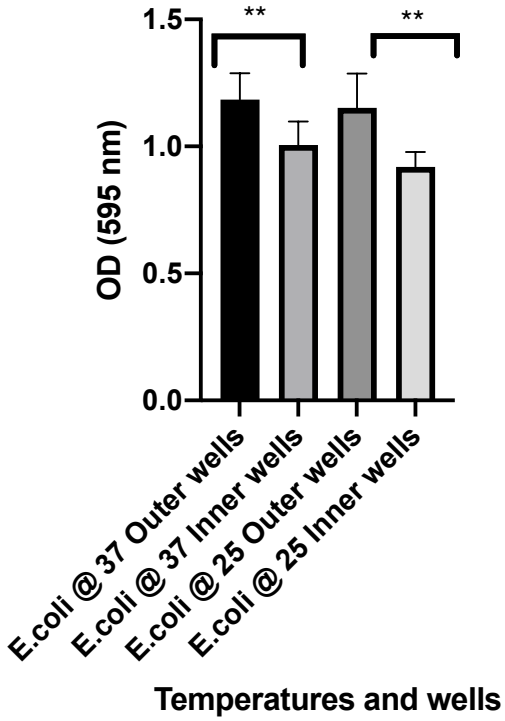


Fig.3.2- Quantitation of planktonic cell density between the innermost and outermost wells of a Co-star 3599 plate by *E. coli* K12 at 37 and 25 degrees Celsius. Replicate number =6. A Mann-Whitney U-test found significance between the two 37°C and 25°C wells (P=0.0087 and P= 0.0043 respectively).

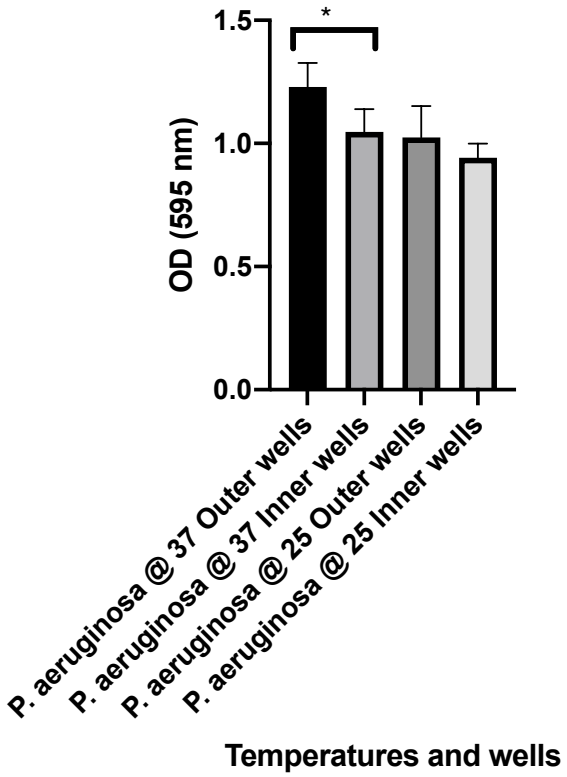


Fig 3.3- Quantitation of planktonic cell density between innermost and outermost wells of a Co-star 3599 plate by *P. aeruginosa* PA01 at 37 and 25 degrees Celsius. Replicate number =6, A Mann-Whitney U-test found significance between the 37°C wells, but not the 25°C wells (P=0.0152 and P=0.484 respectively). Error bars indicate standard error of the mean (SEM).

A mean was taken of absolute OD readings from six wells per bacterial species in either temperature from the innermost and outermost wells of the Co-star 3599 plate. Significant differences between wells can be observed between wells in *E. coli* at both temperatures. In particular, after performing a Mann-Whitney U-test between the innermost and outermost wells at 37 degrees, a significant difference was observed between the wells (P value = 0.0087) and 25 degrees (P value = 0.0043) (Fig. 3.2). Interestingly, in *P. aeruginosa*, a significant difference between the wells was observed at 37 degrees after performing a Mann-Whitney U-test (P value 0.0152), but not at 25 degrees (P=0.484) (Fig. 3.3).

3.2.2 Media and temperature comparisons

The effect of different media, temperature and incubation time was also of interest to this project, to fine-tune experimental conditions. Figure 3.4 shows the optical densities of *E. coli* and *P. aeruginosa* in two different growth media and two different temperatures, 25 and 37 degrees Celsius, after **24 hrs.** incubation. The average OD was calculated by taking an average of six replicate wells from the innermost wells of a Co-star 3599 plate.

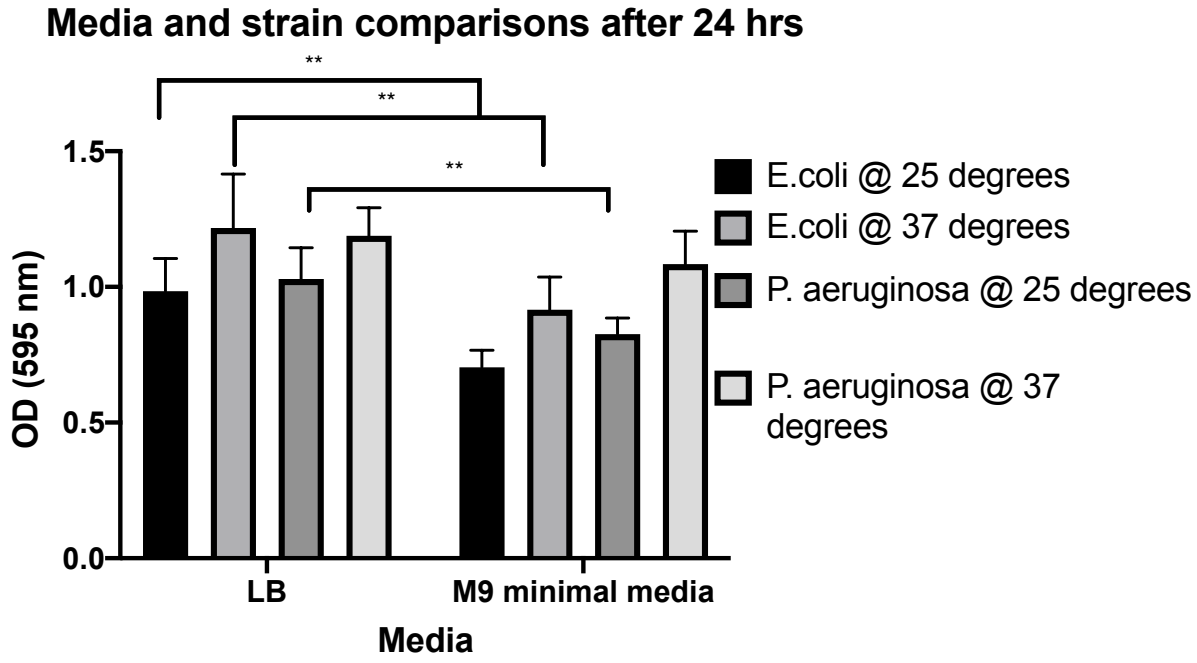


Fig. 3.4- Quantitation of planktonic cell density between *E. coli* K12 and *P. aeruginosa* PA01 grown on nutrient-rich (LB) media and M9 minimal media at two temperatures, 25 and 37°C, measured after 24hrs incubation. Replicate number = 6 and Mann Whitney U-tests found significant differences between groups (P=0.0022, P=0.0087, P=0.0022 and P=0.2900, respectively).

A mean was taken of absolute OD readings from six wells per bacterial species in each temperature and medium from the innermost wells of a Co-star 3599 plate (Fig.3.4). After performing a Mann-Whitney U-test between each growth medium, a significant difference was observed between *E. coli* at 37 degrees (P value = 0.0022), *E. coli* at 25 degrees (P value = 0.0087) and *P. aeruginosa* at 25 degrees (P value = 0.0022). The difference between growth media in *P. aeruginosa* at 37 degrees was classed as non-significant.

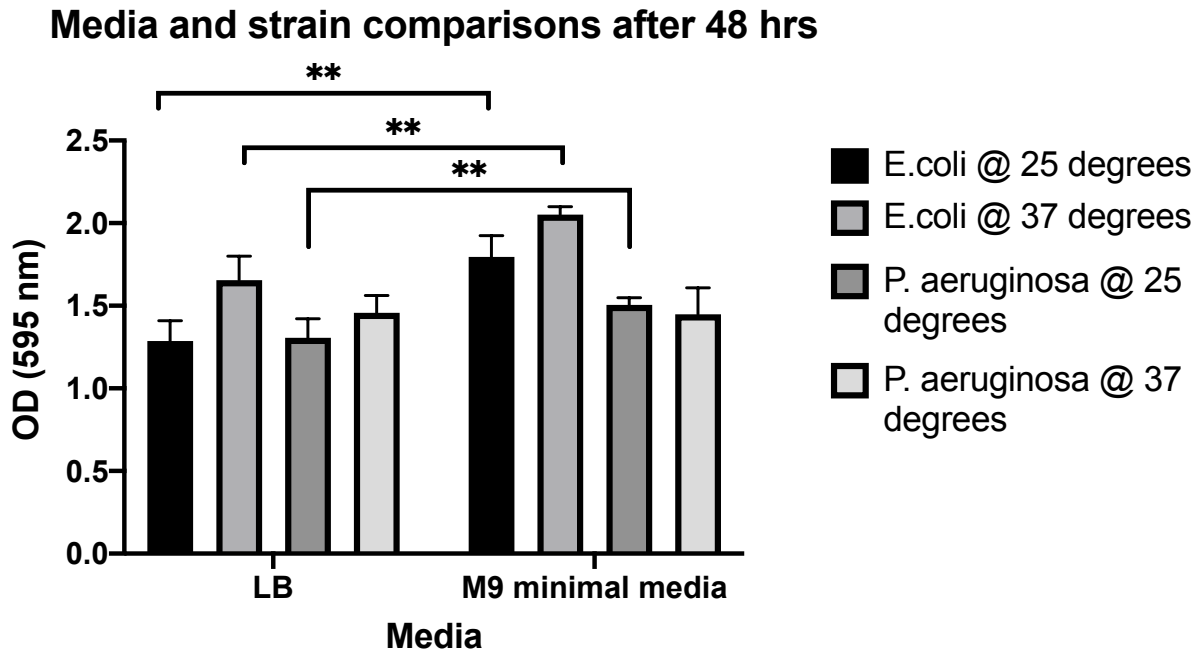


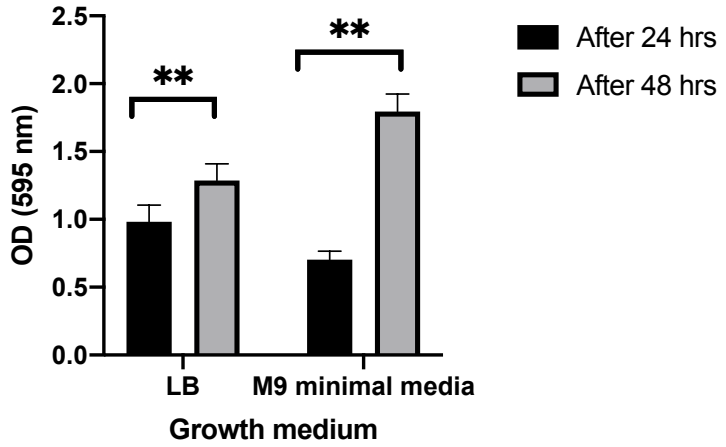
Fig. 3.5- Quantitation of planktonic cell density between *E. coli* K12 and *P. aeruginosa* PA01 grown on nutrient-rich (LB) media and M9 minimal media at two temperatures, 25 and 37°C, measured after 48 hrs. incubation. Replicate number = 6 and Mann Whitney U-tests found significant differences between groups (P=0.0022, P=0.0022, P=0.0087 and P=0.569, respectively).

Fig 3.5 shows the same experiment but after 48h incubation. A mean was taken of absolute OD readings from six wells per bacterial species in each temperature and medium from the innermost wells of a Co-star 3599 plate. After performing a Mann-Whitney U-test between each growth medium, a significant difference was observed between *E. coli* at 37 degrees (P value = 0.0022), *E. coli* at 25 degrees (P value = 0.0022) and *P. aeruginosa* at 25 degrees (P value = 0. 0.0087). The difference between growth media in *P. aeruginosa* at 37 degrees was classed as non-significant.

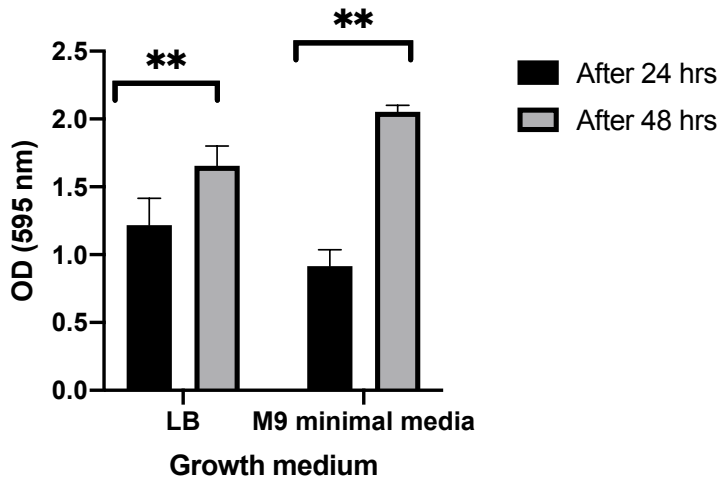
3.2.3 Incubation time comparisons

In addition to 96-well plate geometry, media and temperature, incubation time also plays a large role in influencing planktonic growth. Figures 3.6 A-D shows the optical densities *of E. coli* and *P. aeruginosa* in two different growth media (LB and M9 minimal media), two

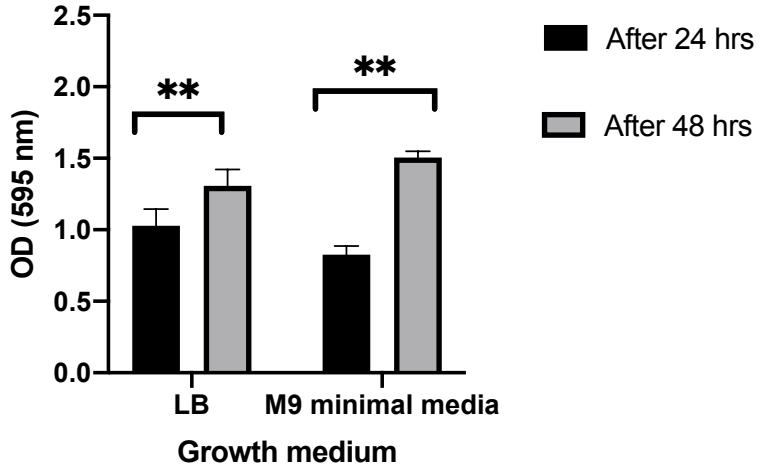
different temperatures, 25 (Fig. 3.6 A and C) and 37 degrees Celsius (Fig. 3.6 B and D), and at two different incubation times, 24 and 48 hours.



A



B



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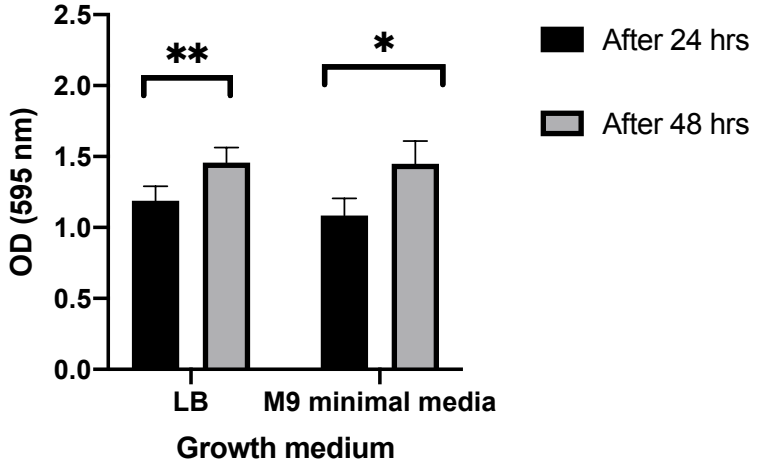


D

Fig.3.6 A-D show the quantitation of planktonic cell densities of *E. coli* (Fig. 3.6 A and B) and *P. aeruginosa* (Fig. 3.6 C and D) after 24 and 48 hrs. Incubation at 25°C (Fig. 3.6 A and C) and 37°C (Fig 3.6 B and D). N=6 for each sample and error bars indicate standard deviation. Mann-Whitney U-tests showed significant differences between incubation times in both media.

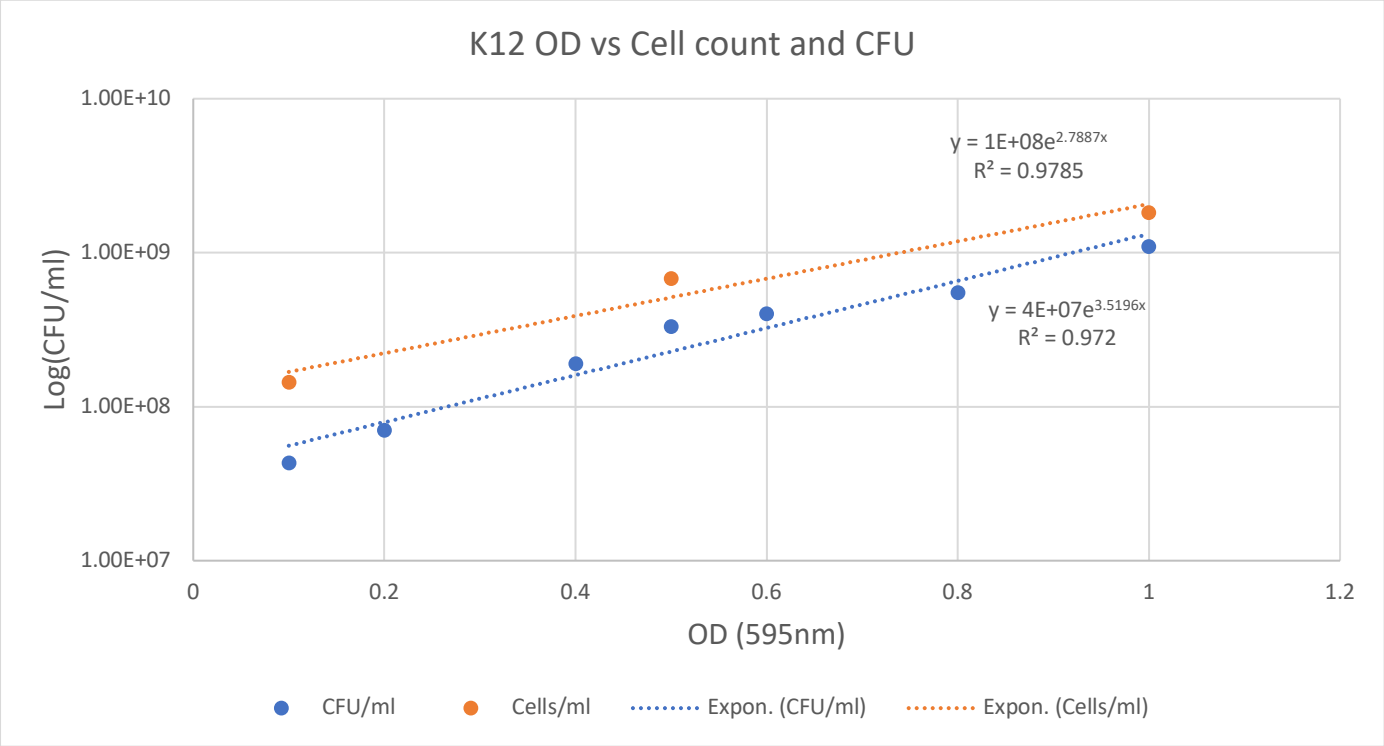
A mean was taken of absolute OD readings from six wells per bacterial species in each temperature and medium, in 24 and 48 hrs. intervals from the innermost wells of a Co-star 3599 plate. After performing a Mann-Whitney U-test between each growth medium, a significant difference in growth was observed between 24 and 48 hrs. incubation across all species and media. *E. coli* exhibits a significant increase in OD at 25 degrees in both LB and M9 minimal media (Fig. 3.6 A) (P-value = 0.0043 and 0.0022, respectively), which is also observed at 37°C after 48hrs (Fig. 3.6 B) (P-value = 0.0087 and 0.0022, respectively). *P. aeruginosa* exhibits a significant increase in OD at 25 degrees in both LB and M9 media (Fig. 3.6 C) (P-value = 0.0087 and 0.0022, respectively). At 37°C, *P. aeruginosa* showed an increased OD in LB medium after 48 hrs. (Fig. 3.6 D) (P-value = 0.0022), but in M9 minimal media although the difference between ODs is still significant, it is lower in comparison with other conditions.

3.2.4 Optical density VS Colony forming units VS Cell counts calibration curve

It has been shown that optical density measurements of microbial growth can vary between species, cell sizes and cell morphologies, which highlights the need for the generation of a

calibration curve for each species to help estimate cell numbers and colony forming units from optical density measurements. (Stevenson et al., 2016). In addition, the generation of Log reduction data for the active oxygen product was an essential part of the project, which also required the generation of calibration curves. This was also of interest to the sponsoring company. The graphs generated cover a range of 3 Logs and the calibration curves cover the range of a 1 log difference between OD=1 and OD=0.1 (Fig. 3.7 A and B). In addition, differences between OD and CFU measurements were found between *E. coli* (Fig. 3.7 A) and *P. aeruginosa* (Fig. 3.7 B), confirming the results of previous studies (Choi et al., 2012; Stevenson et al., 2016).

A



B

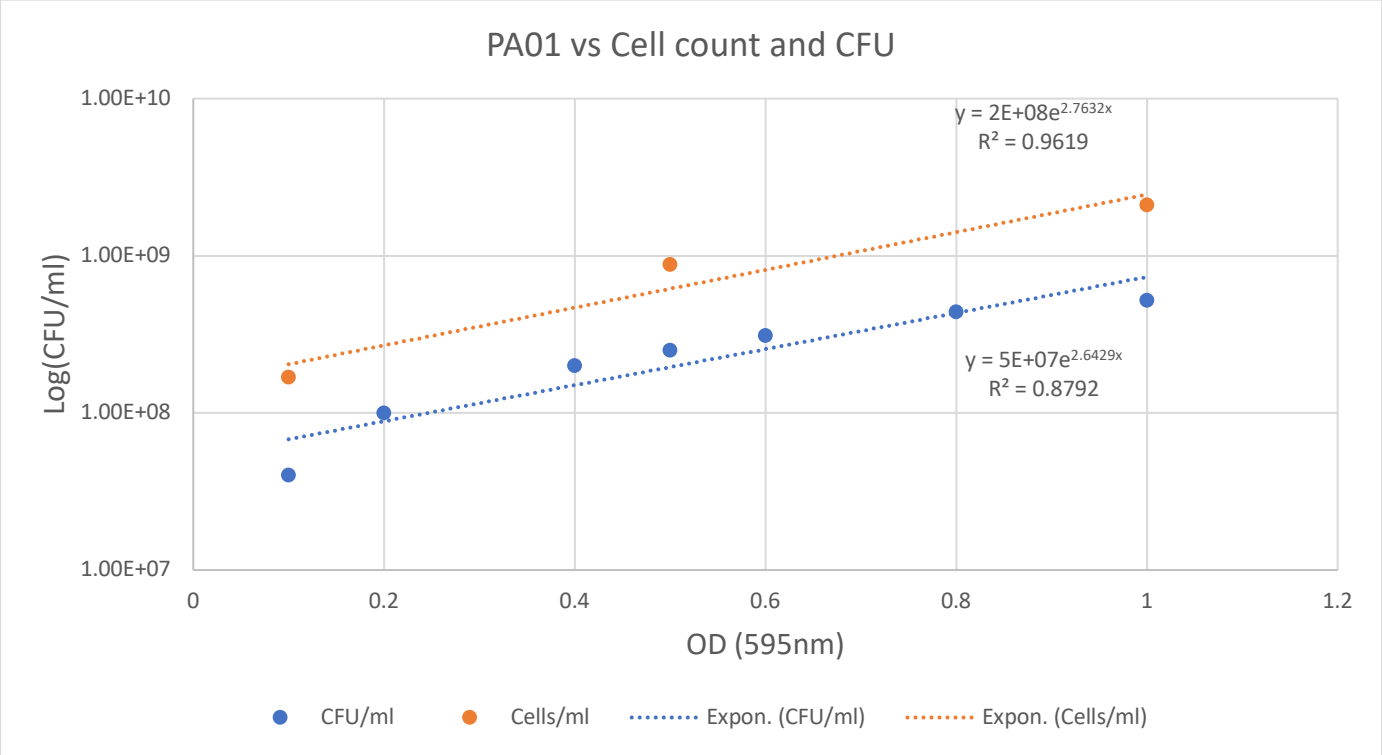


Fig. 3.7 A – B show the calibration curves generated in order to convert OD measurements to CFUs and Cell counts. The ODs and CFUs tested for both *E. coli* and *P. aeruginosa* over a range of 0.1 to 1 AU, while cell counts were measured at ODs of 0.1, 0.5 and 1 AU.

The trendline for cell counts is higher than the curve for CFUs in both graphs (Fig. 3.7A and B), as both viable and non-viable cells were counted. OD measurements below 0.1 were not performed as they tend to be unreliable and are not widely used in studies (Galbán et al., 2007). For ODs below 0.1, the CFU data are most informative. In addition, the results generated come in agreement with research papers on the same topic for both species (Choi et al., 2012; Stevenson et al., 2016)**.** The trendlines generated were used for the conversion of OD measurements to cell counts and CFU and the generation of Log reductions.

3.2.5. Biofilm development – temperature comparisons

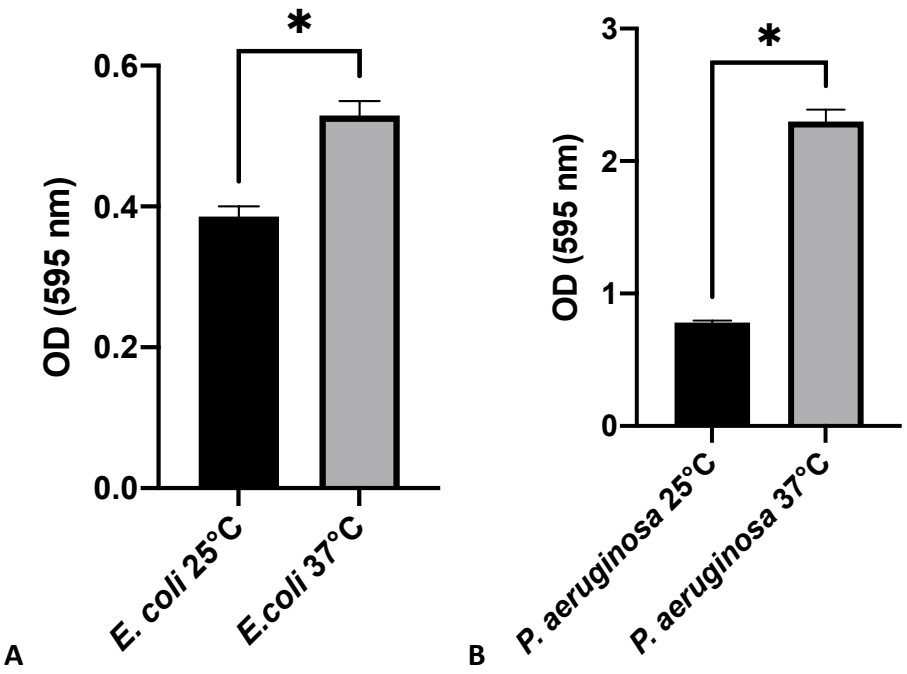


Figure 3.8 A and B: Quantitation of absolute biofilm formation of *E. coli K12* (Fig 3.8 A) and *P. aeruginosa PA01* (Fig. 3.8 B) in M9 media after 24hrs at pH 7.4, in two temperatures (25 and 37°C). N=4 for each bar and error bars indicate standard error of the mean (SEM). Mann-Whitney U-tests showed significant differences between incubation temperatures.

A mean was taken of absolute OD readings from four wells per bacterial species in each temperature from the innermost wells of a Co-star 3599 plate. After performing a Mann-Whitney U-test between each growth medium, a significant difference was observed between *E. coli* at 37 and 25°C (P = 0.0286) and *P. aeruginosa* at 37 and 25°C (P = 0.0286).

3.2.6 Biofilm assay optimized set up

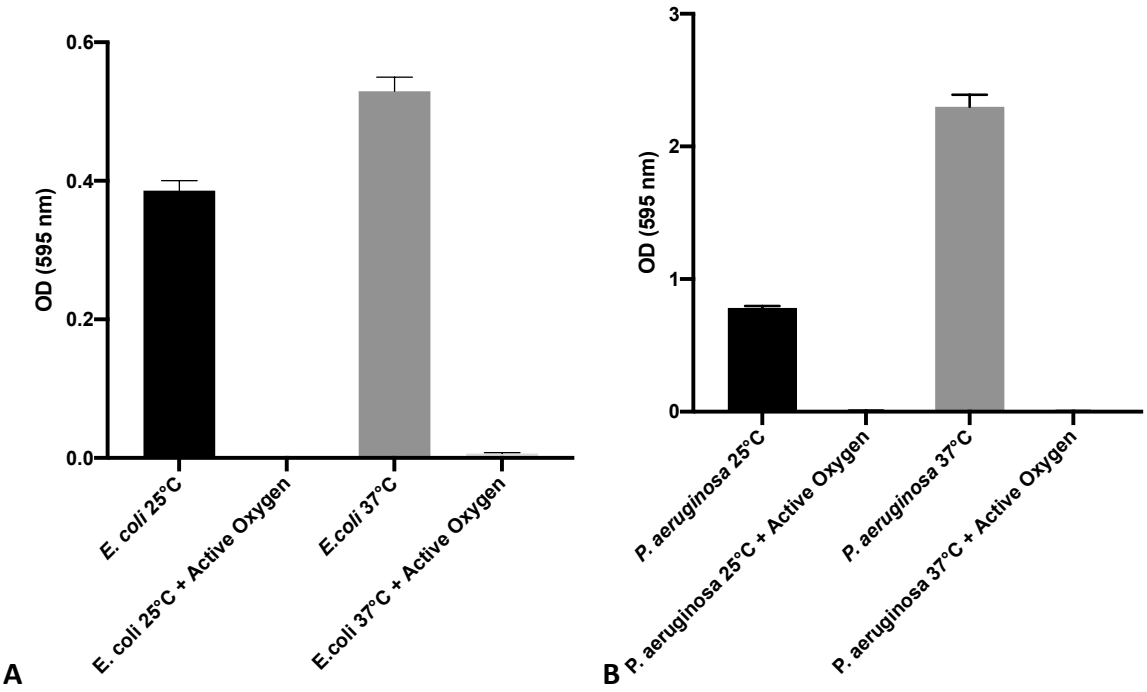
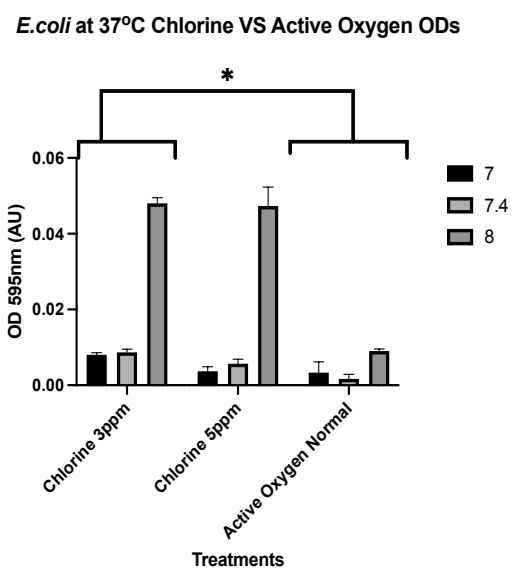


Figure 3.9 A and B: Comparison of absolute biofilm formation of *E. coli K12* (Fig 3.9 A) and *P. aeruginosa PA01* (Fig. 3.9 B) between control and after treatment with Active Oxygen in the SOP concentration diluted in M9 media after 24hrs, pH 7.4, in two temperatures (25 and 37°C). N=4 for controls and N=3 for treated samples and error bars indicate standard error of the mean (SEM).

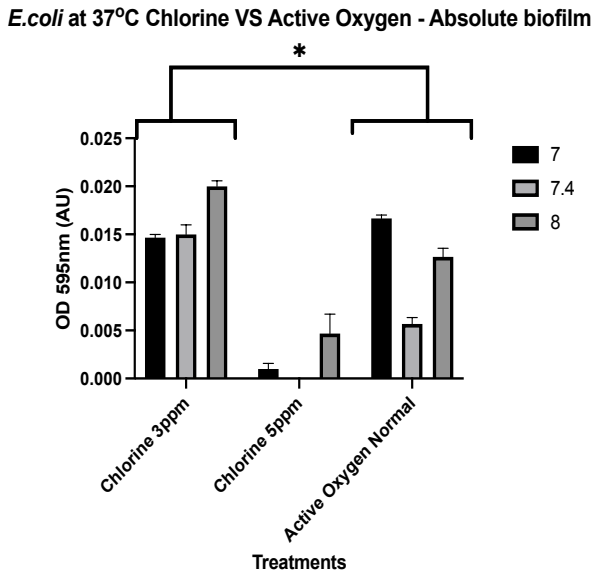
A mean was taken of absolute OD readings from four wells in the controls and three wells in the treated samples. Overall, untreated *P. aeruginosa* exhibited higher biofilm formation in comparison to untreated *E. coli.* Both species exhibited higher levels of biofilm formation at 37°C in comparison to 25°C. No biofilm formation was quantified in the wells treated with active oxygen at the working concentration.

The optimized protocol for biofilm prevention involves the preparation of Active oxygen tablets under the recommended standard operating procedures (SOP), which suggest dissolving the tablets using heated up water (37^{o}C). An equal amount of active oxygen preparation was pipetted together with dilute overnight culture at the same time for a total volume of 200µl. Control wells include only media plus water for a total volume of 200µl. The innermost wells of a Co-star 3599 plate were used. The plates were placed in two different incubators (set up at 25 and 37°C respectively) and the biofilm was quantified using crystal violet staining after 24hrs.

3.2.7 Benchmarking of Active Oxygen against Chlorine



A



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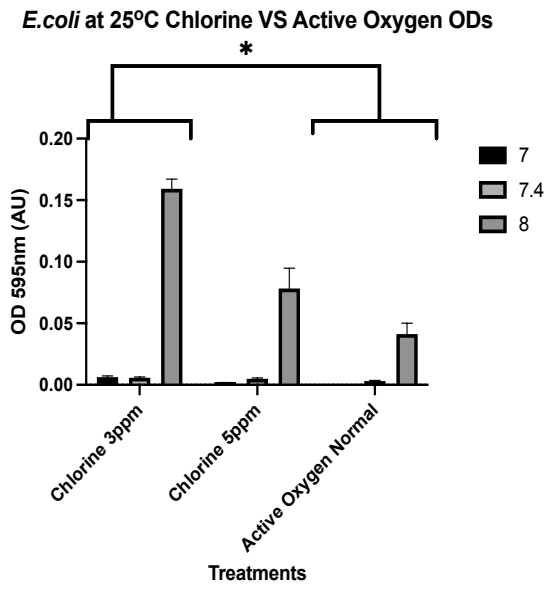

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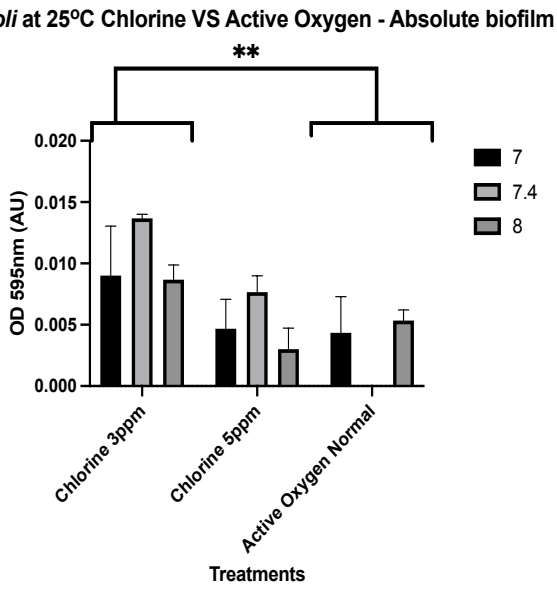


D

Figures 3.10 A-B show the pH effect on efficacies of active oxygen and chlorine treatments of *E. coli* K12. Planktonic cell density (A and C) and absolute biofilm formation (B and D) (both indicated as OD595) of *E. coli* K12 grown in minimal media (M9) at 37°C (A and B) and 25°C (C and D) at pH 7, 7.4 and 8 in the presence of Active Oxygen at 'Normal' concentration (indicating usage at SOP) or in the presence of chlorine at 3 or 5ppm. N=3 for each treatment. Error bars indicate standard error of the mean (SEM). Asterisks indicate significance.

A comparison of Active Oxygen at the recommended concentration against Chlorine, added at the time of inoculation in two concentrations (3ppm and 5ppm) regarding *E. coli* planktonic growth and biofilm prevention at three different pH (7, 7.4 and 8) and two temperatures (25°C and 37°C) was made. The exact values of pH used were chosen as chlorine is known to exhibit maximum bactericidal activity at around pH 7.4 and is degraded at lower or higher pH values (Snoeyink and Jenkins, 1980). Figures 3.10 A-D show the results of benchmarking of active oxygen against two concentrations of chlorine at the optimal pH and pH levels deviating from the advised pH for hot tub maintenance for *E. coli*. At 37°C, very little planktonic growth was observed in the wells treated with the SOP concentration of Active Oxygen (Fig 3.10 A). *E. coli* treated with chlorine at 3ppm in comparison with Active Oxygen showed some planktonic growth which a Mann-Whitney U-test between treatments indicated to be of significance (P = 0.0182). A comparison at pH 8 between chlorine at both concentrations and Active Oxygen showed a significant difference (P = 0.0286 for both 3ppm and 5ppm). This suggests that chlorine loses some of its activity at pH8, while Active Oxygen retains its activity

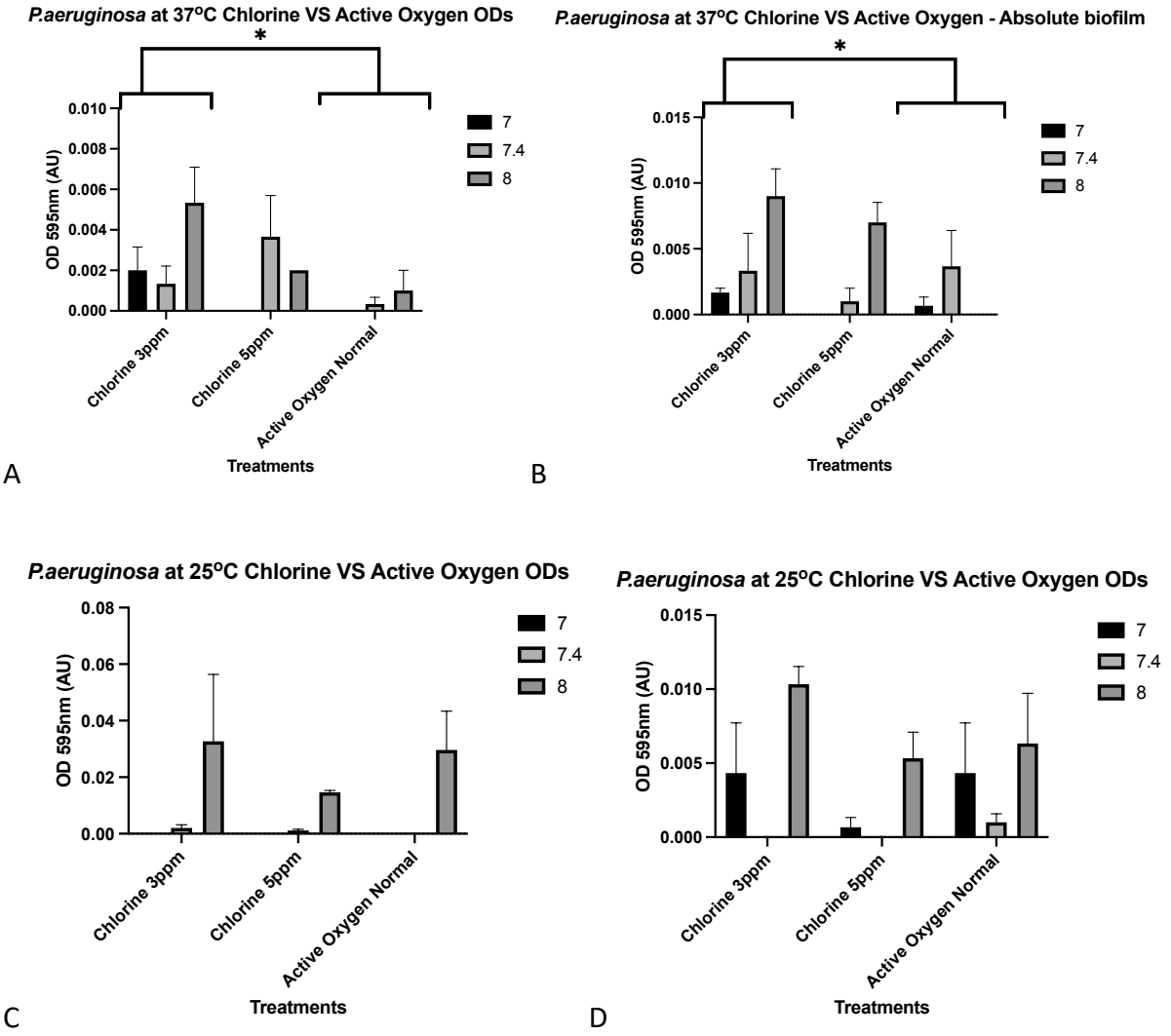
at this pH. The difference in planktonic growth prevention between chlorine at 5ppm and Active Oxygen was found to be non-significant across all pH tested. No significant differences were found between the other pH. From Fig 3.10 A it is clear that active oxygen is better that chlorine at 3ppm at preventing planktonic growth across all pH levels tested.

Regarding absolute biofilm formation at 37^{o}C, low/very low biofilm was observed for all treatments (Fig. 3.10 B). Cells treated with 3ppm of chlorine showed some increased biofilm formation in comparison with Active Oxygen, which a Mann-Whitney U-test confirmed as significant (P = 0.0409). Notably*, E. coli* treated with chlorine at 5ppm exhibited the least biofilm formation, even lower than the cells treated with Active Oxygen (P = 0.0004). Similar to the planktonic growth results, chlorine at 3ppm exhibited some loss of activity at different pH., and when compared to Active Oxygen at pH 8, a significant difference was observed (P = 0.0286). From Figure 3.10 B alone, it can be concluded that chlorine at 5ppm is the best treatment regarding biofilm prevention. However, Active Oxygen is significantly better at biofilm prevention in comparison to chlorine at 3ppm and retains its biofilm preventing properties even at high pH.

At 25^{o}C, very little planktonic growth was observed in the cultures treated with the SOP concentration of Active Oxygen (Fig 3.10 C). *E. coli* treated with chlorine at 3ppm showed some planktonic growth across all pH in comparison with Active Oxygen which a Mann-Whitney U-test between treatments indicated to be of significance (P = 0.0493). The difference between chlorine at 5ppm and Active Oxygen at all pH was observed as non-significant. Following the trend observed at 37^{o}C, chlorine in both concentrations again appears to lose some of its activity in comparison to Active Oxygen at higher pH values. Indeed, at pH 8 a significant difference can be observed between chlorine at 3ppm and Active Oxygen (P = 0.0286), but no significant differences were found across the other pH (7 and 7.4). No significant differences were detected when comparing chlorine at 5ppm and Active Oxygen across pH. Again, it is observed that active oxygen retains its bactericidal properties across the other pH (Fig. 3.10).

Regarding absolute biofilm formation at 25^{o}C, low/very low biofilm was observed for Active Oxygen treatments, and in particular no biofilm at all was measured at pH 7.4. Bacteria treated with 3ppm of chlorine showed some increased biofilm formation in comparison with

Active Oxygen, which a Mann-Whitney U-test confirmed as quite significant (P = 0.0028). The difference between chlorine at 5ppm and Active Oxygen at all pH was calculated as non-significant. The difference between chlorine at 3ppm and 5ppm was calculated as significant (P=0.0148). Interestingly, chlorine at 3ppm appears to lose some of its activity at both low and high pH, and the difference in comparison with Active Oxygen was calculated as quite significant (P=0.028). Overall, it can be observed that Active Oxygen has potent biofilm prevention properties and for the conditions tested its activity is comparable to that of chlorine at 5ppm.



Figures 3.11 A-D show the pH effect on efficacies of active oxygen and chlorine treatments of *P. aeruginosa PA01*. Planktonic cell density (A and C) and absolute biofiom formation (B and D) (both indicated as OD595) of *P. aeruginosa PA01* grown in minimal media (M9) at 37°C (A and B) and 25°C (C and D) at pH 7, 7.4 and 8 in the presence of Active Oxygen at 'Normal'

concentration (indicating usage at SOP) or in the presence of chlorine at 3 or 5ppm. N=3 for each treatment. Error bars indicate standard error of the mean (SEM). Asterisks indicate significance.

Benchmarking of new products/product applications is important to demonstrate similar or better efficacy of the product against products in current use. Here, chlorine was chosen at 3ppm and 5ppm regarding *P. aeruginosa* planktonic growth and biofilm prevention, at three different pH (7, 7.4 and 8) and two temperatures (25°C and 37°C). Chlorine products for the hot tub market were also obtained from the sponsoring company, including recommendations on concentrations and pH. Figure 3.11 A shows the results of benchmarking active oxygen against 2 concentrations of chlorine at the optimal pH and pH levels deviating from the advised pH for hot tub maintenance for *P. aeruginosa*. Similar to the results for *E. coli K12* at 37^{o}C (Fig. 3.11 A), very little planktonic growth was observed from the bacteria treated with the working concentration of Active Oxygen across all pH. Wells treated with chlorine at 3ppm showed some planktonic growth which a Mann-Whitney U-test between chlorine at 3ppm and Active Oxygen indicated to be of significance (P = 0.0182). The difference between chlorine at 5ppm and Active Oxygen was observed to be of no significance across all pH. Chlorine at 3ppm appears to lose some of its activity at both high and low pH in comparison to Active Oxygen, however the differences in comparison to Active Oxygen after performing a Mann-Whitney U-test were observed as non-significant, even at pH=8.

Regarding absolute biofilm formation at 37^{o}C (Fig. 3.11 B), low biofilm production was observed by the bacteria treated with the working concentration of Active Oxygen. Wells treated with 3ppm of chlorine showed increased biofilm formation in comparison with Active Oxygen, which a Mann-Whitney U-test confirmed as significant (P = 0.0359). The difference in biofilm formation between bacteria treated with chlorine at 5ppm and Active Oxygen was calculated to be non-significant across all pH. Importantly, no biofilm formation at all was observed in the wells treated with Active Oxygen at pH 8. In contrast, some biofilm was observed at pH 8 by the bacteria treated with chlorine at 3ppm, and in comparison with Active Oxygen at pH 8, a Mann-Whitney U-test which showed that the difference was significant (P

= 0.0286). It can be thus observed that Active Oxygen has potent biofilm prevention properties against *P. aeruginosa* at 37^{o}C.

At 25^{o}C (Fig. 3.11 C), very low planktonic growth was observed across all treatments and pH, with any growth observed mostly by the bacteria treated at pH 8. However, when compared to Active Oxygen at all pH, both chlorine concentrations showed a non-significant difference. Therefore, regarding *P. aeruginosa* at 25^{o}C, Active Oxygen is no better than both concentrations of chlorine at all pH.

Observations made regarding absolute biofilm formation follow a similar trend (Fig. 3.11 D). No significant difference in biofilm formation was observed between both chlorine concentrations and Active Oxygen at all pH. Some loss of activity at pH 8 can be observed for all treatments, however statistical analysis did not show a significant difference. Therefore, the biofilm prevention properties of Active Oxygen at these conditions are tied with those of chlorine at both concentrations.

3.3 Discussion

Experiments on edge effect in this project showed that indeed, there is a significant difference between the outermost and innermost wells of a 96-well plate. In addition, it was noted that bacteria exhibit higher planktonic growth in LB media compared to M9 after 24hrs, but after 48hrs bacteria exhibited higher planktonic growth in M9 media. *E. coli* exhibits a significant increase in OD at 25 degrees in both LB and M9 minimal which is also observed at 37°C after both 24 and 48 hrs. *P. aeruginosa* exhibits a significant increase in OD at 25 degrees in both LB and M9 media after 24 and 48 hrs. At 37°C, P. aeruginosa showed an increased OD in LB medium after 48 hrs. Calibration curves generated for both species will allow conversion of OD to CFUs and cell counts. Benchmarking of Active Oxygen against chlorine showed that Active Oxygen remains relatively stable across all pH and especially pH 8 and its activity can be comparable to chlorine at 3ppm.

Optical density measurements in either 25°C or 37C from the innermost and outermost wells of the Co-star 3599 plate showed significant differences in planktonic growth between wells can be observed between wells in *E. coli* at both temperatures. In *P. aeruginosa*, a significant

difference between the wells was observed at 37°C but not at 25°C. This comes in agreement with previous studies which have demonstrated similar effects (Lundholt et al., 2003). Therefore, it was decided to exclude the outermost row of wells for this project.

Regarding media comparisons, it was observed that after 24hrs incubation, the highest planktonic growth was noted in the LB media, with the exception of *P. aeruginosa* at 25°C. After 48hrs incubation, it was noted that both *E. coli* and *P. aeruginosa* showed higher planktonic growth in M9 minimal media in comparison to LB after 48hrs incubation (Fig. 3.4 and 3.5). This may be because of *E. coli* and *P. aeruginosa* stress tolerance (Jorgensen et al., 1999). Stress tolerance has been shown to provide *E. coli* and *P. aeruginosa* with tolerance to prolonged starvation in glucose-supplied media, such as the M9 which may explain the increase in growth (Jorgensen et al., 1999).

Regarding time comparisons, *E. coli* showed an increase in OD at 25 degrees in both LB and M9 minimal which is also observed at 37°C after both 24 and 48 hrs. *P. aeruginosa* showed a significant increase in OD at 25 degrees in both media after 24 and 48 hrs. At 37°C, P. aeruginosa showed an increased OD in LB medium after 48 hrs. Prolonged starvation in the stationary phase, which could be the case for results obtained after 48hrs incubation, has also been shown to result in stress response. It has been shown that prolonged starvation may provide a growth advantage (Zambrano and Kolter, 1996). This allows the cells to keep growing under starvation, and eventually allow them to proliferate (Jaishankar and Srivastava, 2017). *E. coli* and *P. aeruginosa* exhibit RpoS-mediated stress tolerance which is known for providing tolerance to prolonged starvation in glucose-supplied media, which may explain the increase in growth in results obtained after 48 hrs. (Jorgensen et al., 1999). Futhermore, the Growth Advantage in Stationary Phase (GASP) phenotype, present in both *E. coli* and *P. aeruginosa,* may provide the bacteria with a growth advantage in prolonged starvation, as it allows them to use the nutrients released by dying cells, which may further explain the results from these graphs (Zambrano and Kolter, 1996).

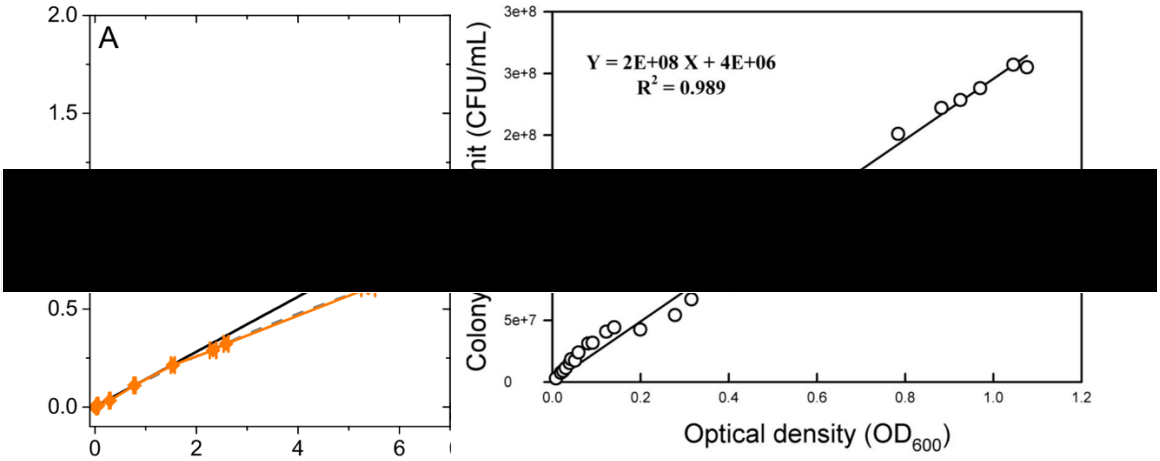
The microtiter plate biofilm assay is widely used for the study of planktonic growth and biofilms, coupled with crystal violet staining to perform biofilm quantification. The 96-well interface allows for the simultaneous screening of a large number of bacterial strains or species and different treatments/growing conditions, in addition to be an inexpensive and

high throughput method (O'Toole, 2011; Azeredo et al., 2016). Additionally, previous research has shown that biofilm data obtained with the microtiter plate assay appeared more replicable and consistent compared to alternative methods, such as microscopic analysis (Djordjevic et al., 2002). However, a drawback of the microtiter plate method is the wide variety of plates provided by the supplier. With a wide variety of pre-treatments, materials and well-shapes available, it is no wonder that different plates can provide varying results. For example, well surface roughness can affect the consistency of the biofilm assays and can lead to higher biofilm formation in *Pseudomonas fluorescens* (Bordeleau et al., 2018). Additionally, culture treated microtiter plates have been used in previous research including *P. aeruginosa* and *E. coli* (Das et al., 2016; Nizet et al., 1998). It is also important to note the shape of the bottom of the wells; flat bottomed wells were ideal for this project as they allowed for maximum light transmission and OD measurements from bottom-reading spectrophotometers such as those used in the experiments described in later chapters (Sittampalam GS, 2004).

The plates used in this project were Co-star 3599. Previous research has shown that the Co-star 3599 plates provided more consistent readings and replicable results for both *E. coli* K12 and *P. aeruginosa* PA01 strains compared to plates provided by other manufacturers (Delaney, 2019). Additionally, the provision of a sterile polystyrene lid allowed for simpler handling, reduced risk of contamination and less condensation compared to sterile sealant use, which also allows for more accurate readings and consistent results (Delaney, 2019).

It has been shown that optical density measurements of microbial growth can vary between species, cell sizes, cell morphologies and stress responses, which makes it important to generate a calibration curve for each organism to help estimate cell numbers and colony forming units from optical density measurements (Stevenson et al., 2016). The graphs generated covered a range of 3 Logs and the calibration curves covered the range of a 1 log difference between OD=1 and OD=0.1 (Fig. 3.7 A and B). The differences between OD and CFU measurements between *E. coli* (Fig. 3.7 A) and *P. aeruginosa* (Fig. 3.7 B) evidence the findings of previous studies (Stevenson et al., 2016).The trendline for cell counts is higher than the curve for CFUs in both graphs (Fig. 3.7A and B), as both viable and non-viable cells were counted. In addition, the results generated come in agreement with research papers on

the same topic for both species (Stevenson et al., 2016). Most importantly, the findings of this study come in agreement with the findings of two previous studies: For *E.* coli, the growth curve is similar to the Figure 3.10 A, while for *P. aeruginosa* it resembles the growth curve of Figure 3.10 B.



Figures 3.12 A and B: Figure A shows the OD vs Cell concentration of *E. coli* in M9 media (Stevenson et al., 2016) and Figure B shows the CFU vs OD curve of *P. aeruginosa* in LB media (Dong-ju Kim, 2012).

Stevenson et al. showed in their paper that optical density measurements is not proportional to the cell number in a sample and offered some considerations to improve the accuracy of OD measurements. Some important factors which may affect OD measurements include growth and stress conditions, refractive index and cell size. Moreover, the paper presented different OD measurements obtained by five different spectrophotometers for the same sample and found rather shocking differences between brands and models, even after path length correction was applied (Stevenson et al., 2016).

Regarding path length, many OD measurements were taken using plastic disposable cuvettes with a 1cm path length, which differs from the path length in microtiter plates. As the path length in microtiter plates is not fixed, many plate readers, including the FluoStar BMG plate reader used in this project, employ built-in pathlength correction. Pathlength correction normalizes absorbance values measured on a microtiter plate to match the absorbance values measured in a 1cm cuvette.

Regarding chlorine, the two concentrations recommended to use in experiments by the company (3ppm and 5ppm) were the two concentrations used in hot tubs and swim spas. In the experiments in the project, chlorine did not show a significant loss of activity at either pH 7 or pH 8, however, when compared with Active Oxygen at the normal dilution, Active Oxygen exhibited higher stability and statistically significant higher activity. However, it should be noted that for both treatments, very low endpoint readings were obtained.

In addition, the Active Oxygen exhibited stability across all pH tested, even at pH 8. This is of importance, as the tap water in some regions of the UK has been found to be in the alkaline range and commonly treated to be in the basic pH range (Ander et al., 2016). Alkaline pH is known to degrade chlorine, which is important as it exhibits reduced sanitizing efficacy at high pH values, which may become an issue in the hot tub environment (Wahman, 2018). In addition, sunlight further degrades chlorine, contributing to the loss of activity (Jain et al., 2010). The stability of Active Oxygen under basic pH and its resistance to sunlight degradation and long half-life make it an attractive alternative to chlorine (ECHA, 2020).

Chapter 4: Efficacy testing of Active Oxygen and Water Conditioner and their combinations on bacterial growth and biofilm formation under static conditions

4.1 Introduction

The aim of this chapter was to assess the efficacy of the products for water sanitation and biofilm removal and prevention and their suggested combinations in the Co-star 3599 polystyrene 96 well plates. The protocols for static biofilm analysis are provided in the the materials and methods chapter. The first product is Eco3Spa Hot Tub Cleaner (Product 1) It is advertised as an "environmentally friendly biofilm remover and its suggested use is once every three months. The second product is Eco3Spa Water Conditioner (Product 2), is added in conjunction with the tablets every month. The third product is Eco3Spa Water Sanitizer tablets (Product 3), which utilizes active oxygen to sanitize the water. Product 1 (biofilm remover) and 2 (water conditioner) were used at the recommended (SOP) concentrations, as the company's focus was on active oxygen. A series of dilutions were prepared for the SOP experiments in order to generate a dose-response curve, which was also of interest to the sponsoring company. (Section 2.6.3).

Static biofilm assays were performed with two bacterial strains *E. coli* K12 and *P. aeruginosa* PAO1 after addition of various concentrations and combinations the Biofilm remover, Active Oxygen and Water Conditioner products. These results were then quantified to assess the level of effect that each of the products had upon the prevention of absolute biofilm formation and planktonic growth.

The product efficacy was assessed in both the recommended standard operating procedures (SOP), which suggests product addition to a working hot tub with heated up water (37^{o}C), as well as non-SOP conditions, which include product addition to water at 25°C to provide a series of results and compare the effect of temperature and recommended SOP on product efficacy.

The media selected represent the nutrient rich environment of the hot tub piping (LB) and the minimal environment of the hot tub water (M9). However, due to COVID lab closure and time constraints, it was decided to prioritize M9 media for the SOP experiments as it is more

representative of the environment where the products will be used. The sponsoring company was in agreement with this decision.

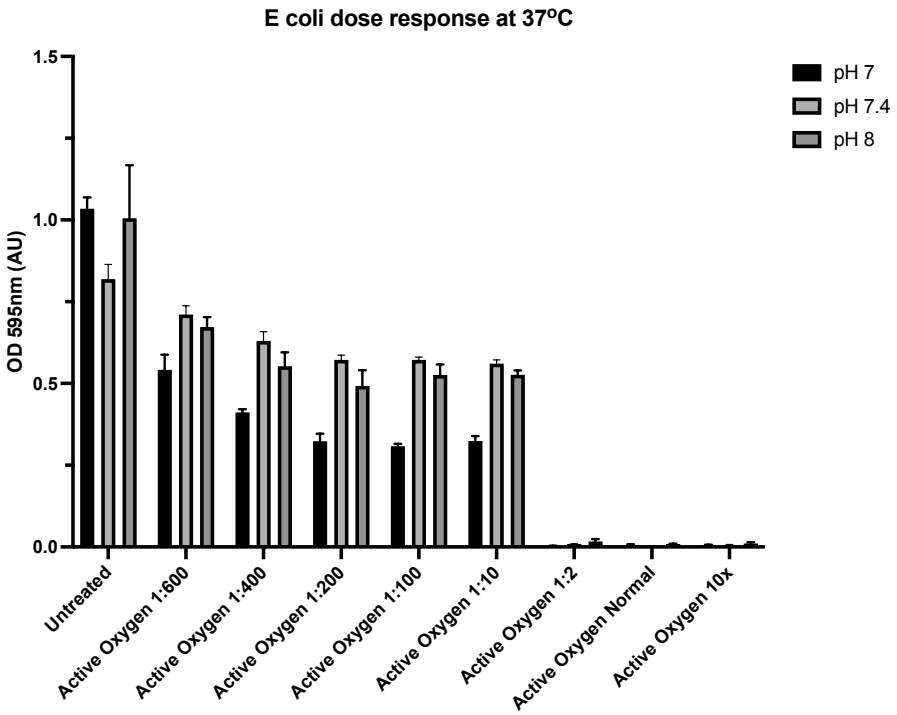
In addition, Eco3Spa requested the generation of a dose-response curve of Active Oxygen including the SOP concentration as well as weaker and stronger concentrations, added at the time of inoculation at three different pH (7, 7.4 and 8) and two temperatures (25°C and 37°C), in order to measure the inhibition of planktonic growth. The exact values of pH used were chosen as the activity of active oxygen under different pH is not known. Additionally, as chlorine exhibits optimal activity at 7.4 and is degraded at the pH extremes, the effect of pH on active oxygen at these three values was assessed in order to compare the products, which was also of great interest to the sponsoring company.

4.2 Results

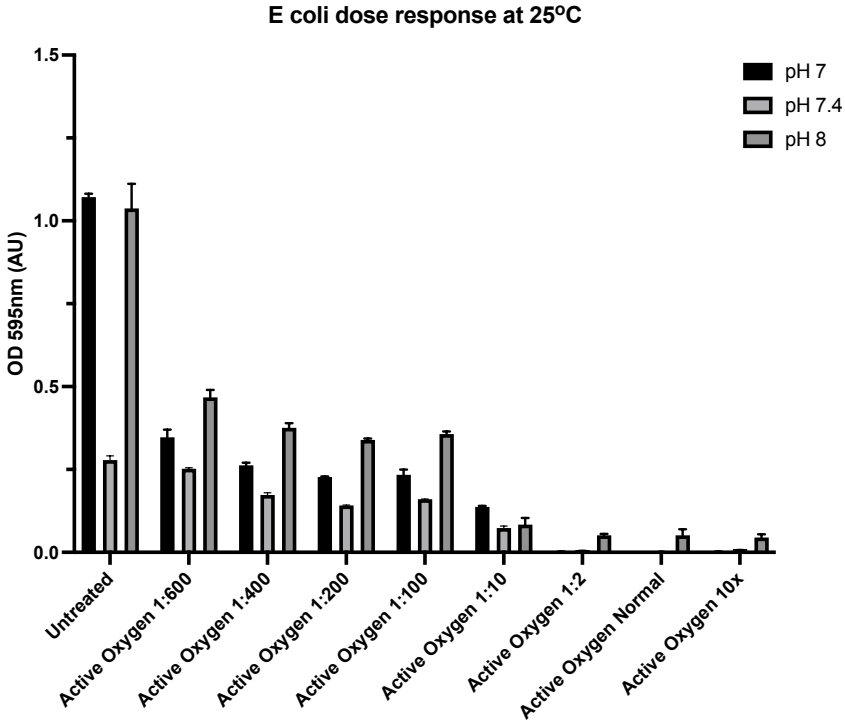
The experiments in this section were performed according to the Recommended Standard Operating Procedures (SOP) (See section 2.6.3)

4.2.1 Efficacy of active oxygen for prevention of planktonic growth in minimal media (M9)

A dose-response curve of Active Oxygen including the SOP concentration as well as weaker and stronger concentrations, added at the time of inoculation at three different pH (7, 7.4 and 8) and two temperatures (25°C and 37°C) was generated, in order to measure the inhibition of planktonic growth.


E coli dose response at 37°C
pH 7
pH 7.4
pH 8
OD 595nm (AU)
1.5
1.0
0.5
0.0
Untreated
Active Oxygen 1:600
Active Oxygen 1:400
Active Oxygen 1:200
Active Oxygen 1:100
Active Oxygen 1:10
Active Oxygen 1:2
Active Oxygen Normal
Active Oxygen 10x
Active Oxygen relative concentration

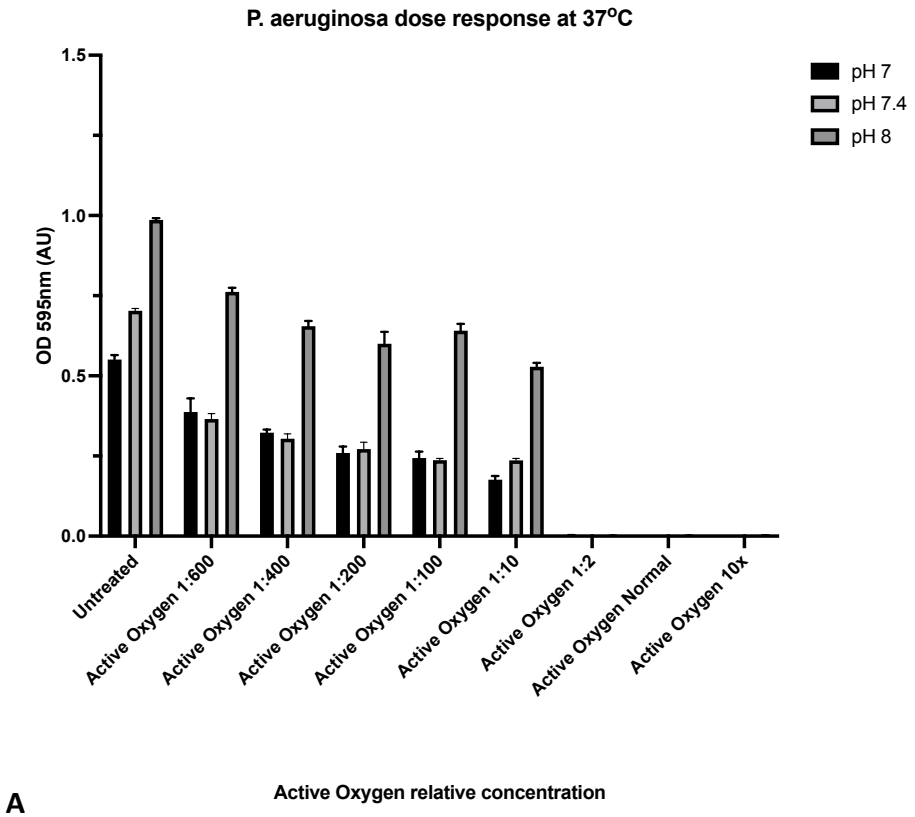
A


E coli dose response at 25°C
pH 7
pH 7.4
pH 8
OD 595nm (AU)
1.5
1.0
0.5
0.0
Untreated
Active Oxygen 1:600
Active Oxygen 1:400
Active Oxygen 1:200
Active Oxygen 1:100
Active Oxygen 1:10
Active Oxygen 1:2
Active Oxygen Normal
Active Oxygen 10x
Active Oxygen relative concentration

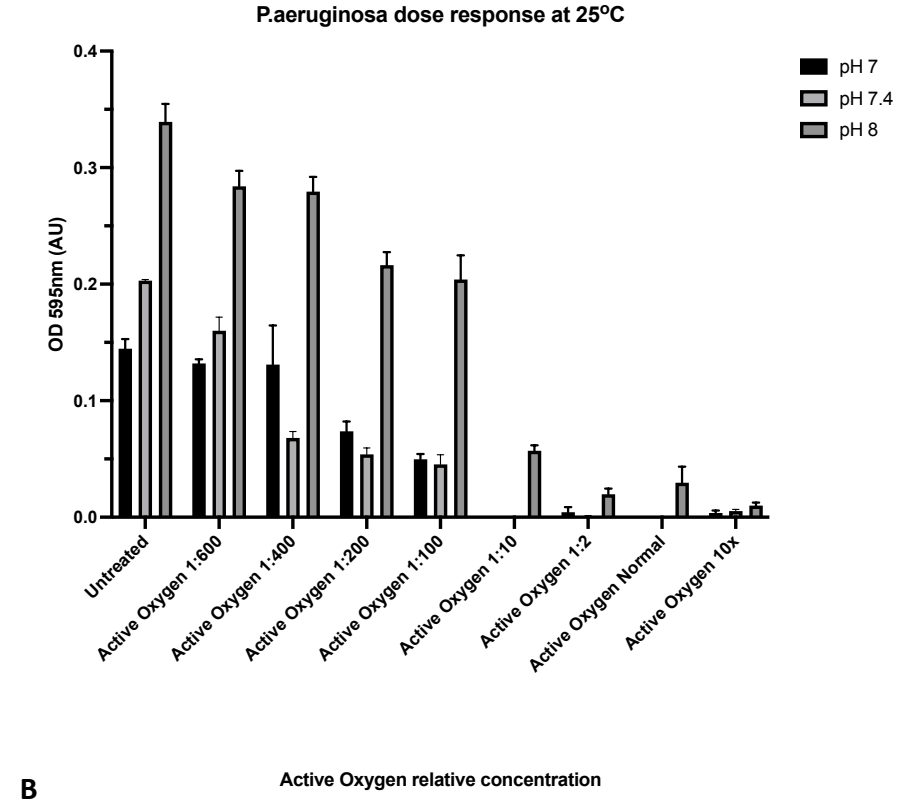
B

Figure 4.1 *E. coli K12* planktonic growth (indicated as OD595) grown in minimal media (M9) at three pHs at 37°C (A) and 25°C (B) in the presence and absence of a range of Active Oxygen concentrations. 'Normal' indicates usage of active oxygen at SOP, other concentrations as indicated relate to normal SOP. N=3 for each data point with the exception of the control (N=4). Error bars indicate standard error of the mean (SEM).

A dose-response curve of Active Oxygen including the SOP concentration as well as weaker and stronger concentrations, added at the time of inoculation at three different pH (7, 7.4 and 8) and two temperatures (25°C and 37°C) was generated, in order to measure the inhibition of planktonic growth, which was of interest to the sponsoring company. The exact values of pH used were chosen as the activity of active oxygen under different pH is not known. In addition, the benchmarking of active oxygen against chlorine, which shows optimal activity at 7.4 and is degraded at lower or higher pH values, was an important objective in this project and of great interest to the sponsoring company (Chapter 3) (Snoeyink and Jenkins, 1980). At 37^{o}C, the highest planktonic growth of *E. coli* in untreated samples (Fig 4.1A Control) was observed at pH=7, followed by pH=8 and finally by pH 7.4. Planktonic growth was supported at 37°C in M9 media containing active oxygen of one-tenth dilution and weaker for all pH tested. Growth was however severely reduced when active oxygen was present at half the SOP normal concentration and stronger at all pHs tested. Across all dilutions, the optical density stayed at a consistent level below 0.700 AU. A similar trend was observed at 25^{o}C, with growth being supported in media containing active oxygen of one-tenth SOP dilution and weaker for all pH tested. Very little planktonic growth observed in media containing active oxygen of one-tenth dilution and stronger. Across all dilutions, the optical density remained at a consistent level below 0.5 AU. Interestingly, there is some, although little, planktonic growth observed at concentrations stronger than one-tenth SOP in pH 8. For *E. coli*, the levels of planktonic growth present in treated wells were lower at 25^{o}C compared to 37^{o}C.



A



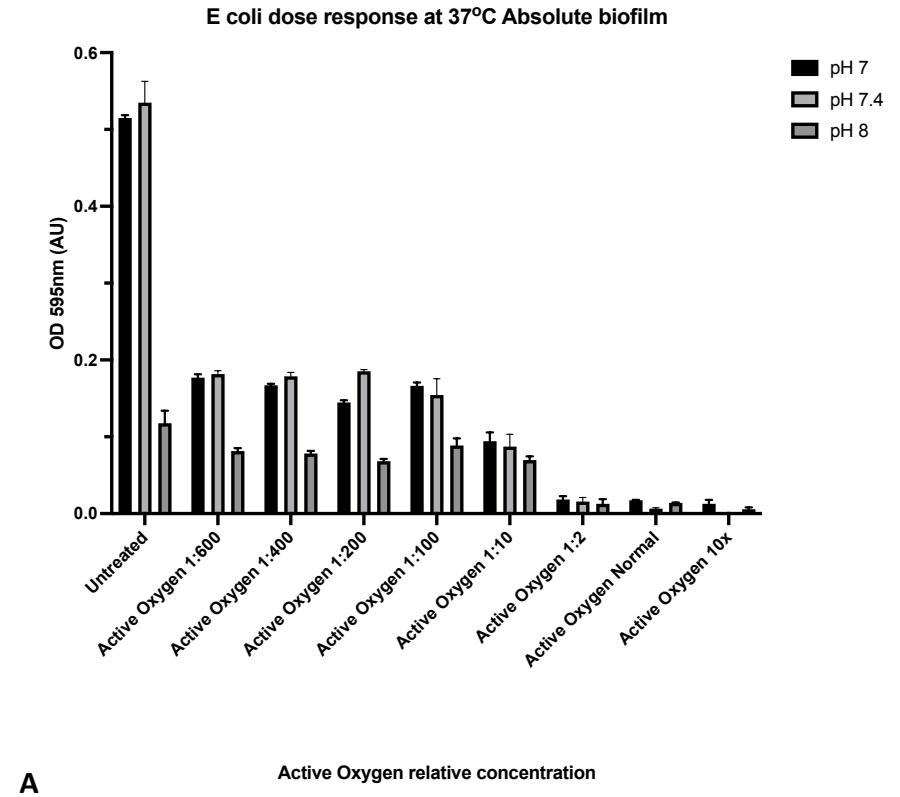
B

Figure 4.2 *P. aeruginosa PA01* planktonic growth (indicated as OD595) grown in minimal media (M9) at three pHs at 37°C (A) and 25°C (B) in the presence and absence of a range of

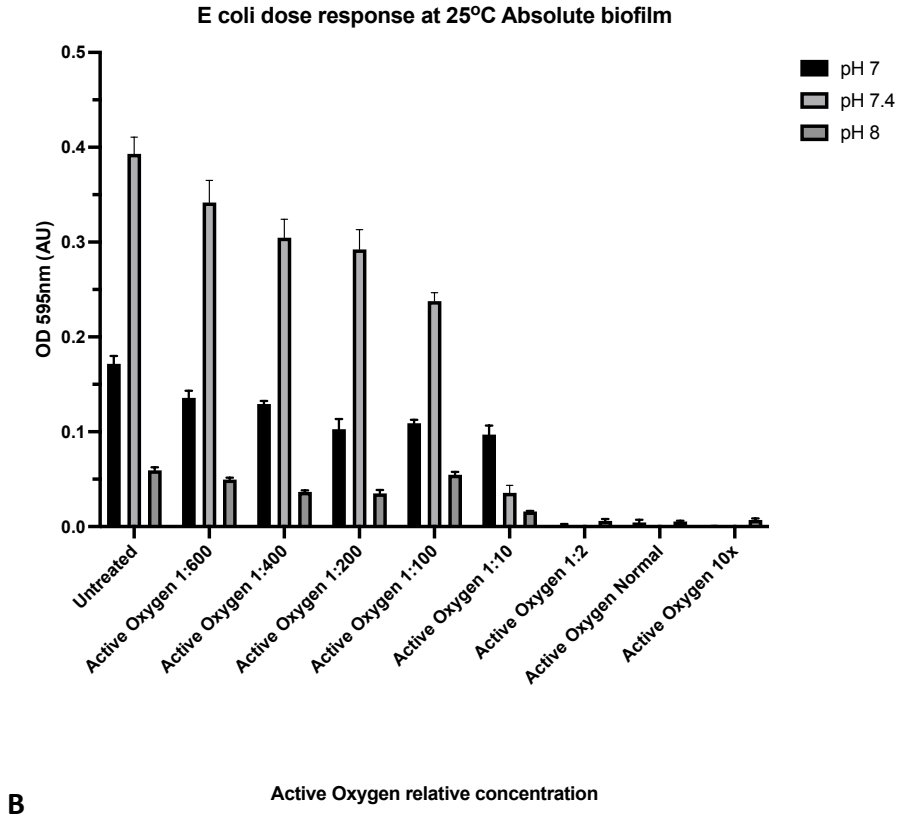
Active Oxygen concentrations. 'Normal' indicates usage of active oxygen at SOP, other concentrations as indicated relate to normal SOP. N=3 for each data point with the exception of the control (N=4). Error bars indicate standard error of the mean (SEM).

A similar dose-response was observed for *P. aeruginosa* cultured in M9 (Fig 4.2). At 37^{o}C, the highest planktonic growth of *P. aeruginosa* in untreated samples (Figure 4.2 A Control) was observed at pH=8, followed by pH 7.4 and pH 7, and a similar trend is observed at 25^{o}C. However, at 37^{o}C, much higher levels of planktonic growth were achieved, which is evident by the scaling on the y axis. In a similar fashion to the trend observed with *E. coli,* planktonic growth was supported at 37°C in M9 media containing active oxygen of one-tenth dilution and weaker for all pH tested, with no planktonic growth observed at all at lower concentrations. Across all concentrations, the optical density remained at a consistent level below 0.800 AU. At 25^{o}C, untreated samples followed a similar growth trend to those at 37^{o}C, with growth being supported in media containing active oxygen of one-tenth SOP dilution and weaker for all pH tested. Again, concentrations weaker than one tenth SOP supported planktonic growth and little growth was observed in higher concentrations. The optical density remained consistent below 0.300 AU. Interestingly, at pH 8, some planktonic growth can be observed even at the highest concentrations, with the exception of 10x concentration. A small spike in optical density can be observed in the 1:400 dilution, but the noticeable error bars must also be noted. For *P. aeruginosa,* the levels of planktonic growth present in treated as well as untreated wells were consistently lower at 25^{o}C compared to 37^{o}C.

4.2.2 Efficacy of active oxygen for prevention of static biofilm in minimal media (M9)



A

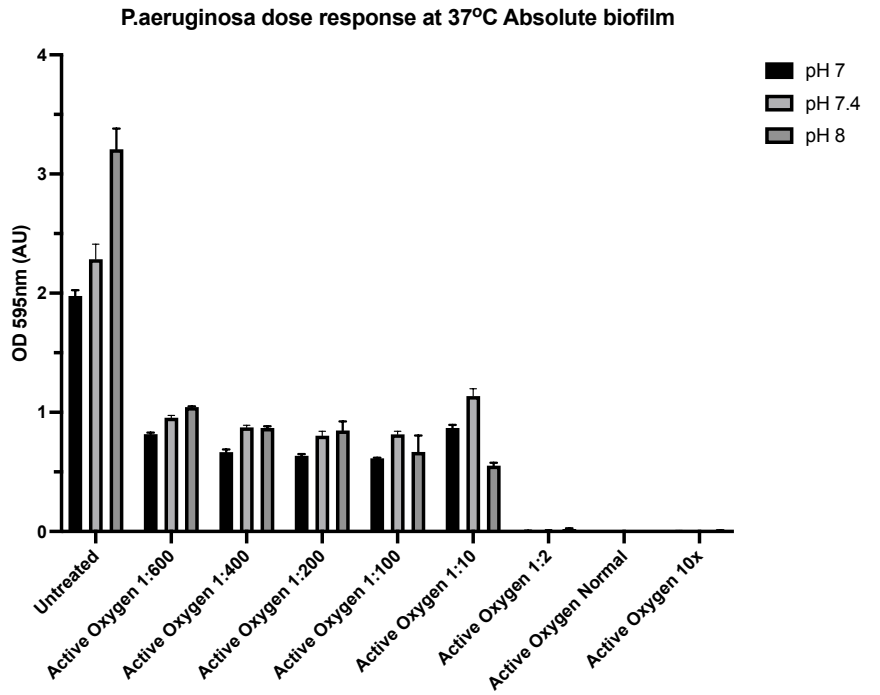


B

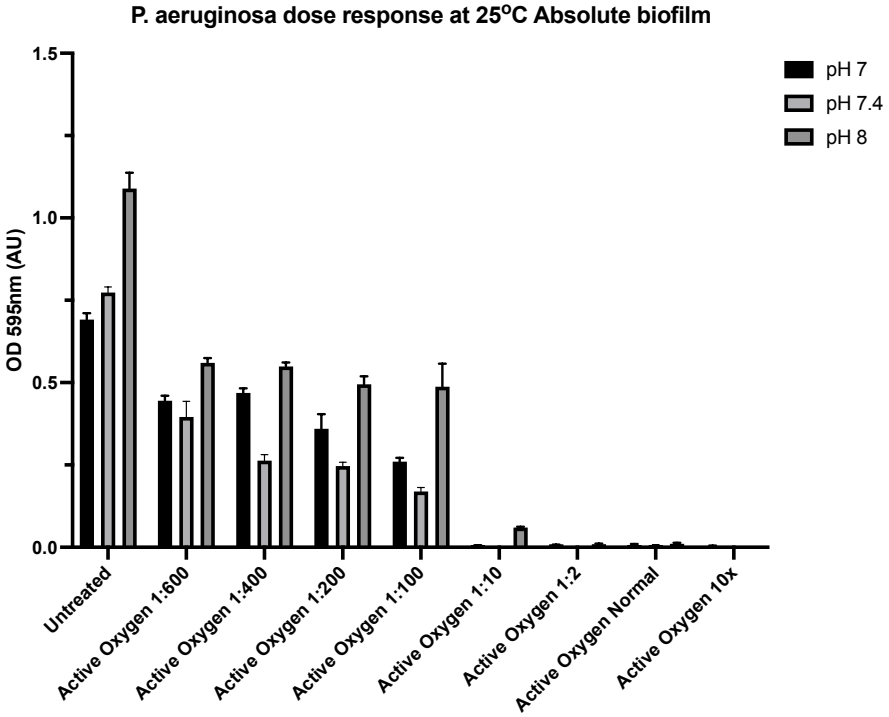
Figures 4.3 A-B *E. coli K12* absolute biofilm formation (indicated as OD595) when grown in minimal media (M9) at three pHs at 37°C (A) and 25°C (B) in the presence and absence of a

range of Active Oxygen concentrations. 'Normal' indicates usage of active oxygen at SOP, other concentrations as indicated relate to normal SOP. N=3 for each data point with the exception of the control (N=4). Error bars indicate standard error of the mean (SEM).

In order to thoroughly assess the biofilm prevention properties of active oxygen, a dose-response curve of Active Oxygen was generated, which included the SOP concentration as well as weaker and stronger concentrations, added at the time of inoculation at three different pH (7, 7.4 and 8) and two temperatures (25°C and 37°C). The dose-response curve was also of interest to the sponsoring company. For *E. coli* grown at 37^{o}C, the highest absolute biofilm formation was observed in untreated wells at pH 7.4, followed by pH 7, and finally pH 8 (Fig 4.3 A). A sharp decline in biofilm formation was observed for the lowest Active Oxygen dilution (1:600) tested, which trend was continued until the active oxygen of one-tenth SOP dilution. Almost no quantifiable biofilm could be observed when half-strength (1:2) or higher concentrations of active oxygen were added. Across all active oxygen dilutions, absolute biofilm remained consistently below 0.200 AU. At 25^{o}C, a slower decrease in biofilm formation could be observed after treatment with Active Oxygen (Fig 4.3 B). Interestingly, biofilm formation decreased sharply at pH 7.4 one-tenth SOP dilution can be seen. Also, at 25^{o}C at the highest concentrations (1:2, Normal and 10x), the levels of biofilms observed were even lower than those at 37^{o}C. Across all concentrations, absolute biofilm remained below 0.380 AU.


P.aeruginosa dose response at 37°C Absolute biofilm
pH 7
pH 7.4
pH 8
OD 595nm (AU)
0
1
2
3
4
Untreated
Active Oxygen 1:600
Active Oxygen 1:400
Active Oxygen 1:200
Active Oxygen 1:100
Active Oxygen 1:10
Active Oxygen 1:2
Active Oxygen Normal
Active Oxygen 10x
Active Oxygen relative concentration

A

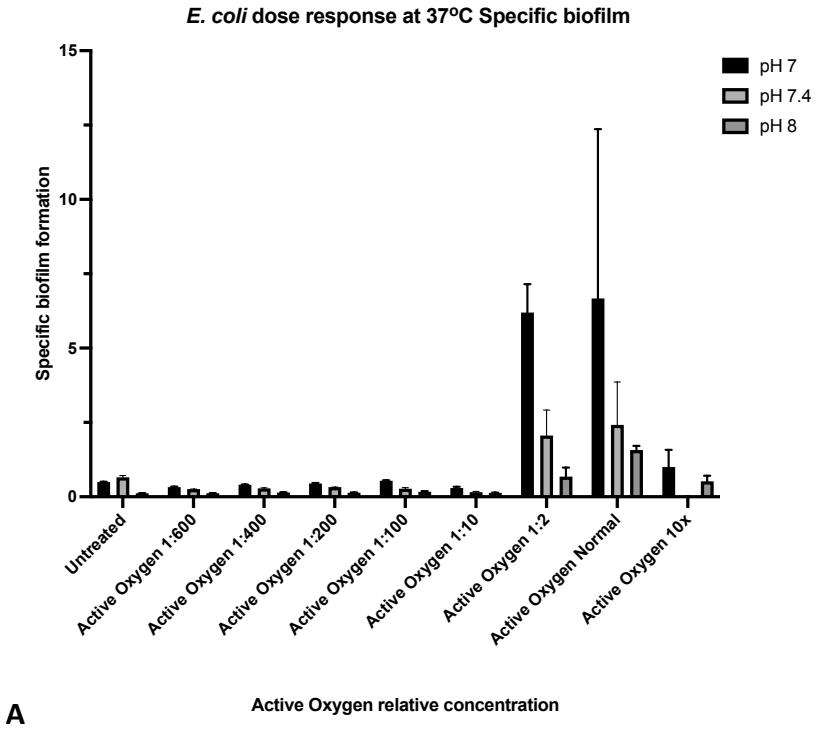

P. aeruginosa dose response at 25°C Absolute biofilm
pH 7
pH 7.4
pH 8
OD 595nm (AU)
0.0
0.5
1.0
1.5
Untreated
Active Oxygen 1:600
Active Oxygen 1:400
Active Oxygen 1:200
Active Oxygen 1:100
Active Oxygen 1:10
Active Oxygen 1:2
Active Oxygen Normal
Active Oxygen 10x
Active Oxygen relative concentration

B

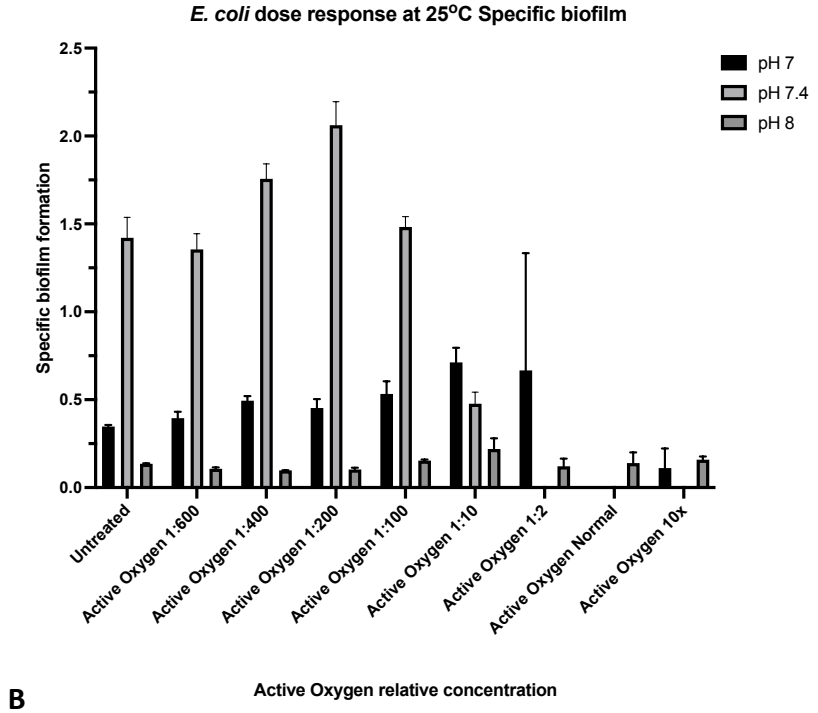
Figures 4.4 A -B *P. aeruginosa PA01* absolute biofilm formation (indicated as OD595) when grown in minimal media (M9) at three pHs at 37°C (A) and 25°C (B) in the presence and absence of a range of Active Oxygen concentrations. 'Normal' indicates usage of active oxygen at SOP, other concentrations as indicated relate to normal SOP. N=3 for each data point with the exception of the control (N=4). Error bars indicate standard error of the mean (SEM).

Again, a similar trend was observed for *P. aeruginosa* biofilms formed when grown in M9 (Fig 4.4). At 37^{o}C, the highest biofilm formation was observed in untreated wells at pH 8, followed by pH 7.4 and finally pH 7. A sharp decline in quantifiable biofilm could be observed from the lowest Active Oxygen concentration, and biofilm formation continues to decline until the media containing active oxygen of one-tenth SOP dilution, where, interestingly, a minor peak appears at pH 7 and 7.4, suggesting higher biofilm formation. Across all concentrations, absolute biofilm remained below 1.050 AU. No quantifiable biofilm was observed at concentrations higher than one tenth SOP dilution. At 25^{o}C, biofilm formation in untreated wells follows the same trend as in 37^{o}C, although in lower quantities. Again, a decrease in biofilm formation was observed from the lowest Active Oxygen concentration. A small amount of biofilm is noted at media containing one-tenth SOP dilution, where in higher concentrations no biofilm was quantifiable. Across all concentrations, absolute biofilm remained below 0.600 AU.

4.2.3 Efficacy of active oxygen for prevention of static biofilm in minimal media (M9)– Specific biofilm formation



A

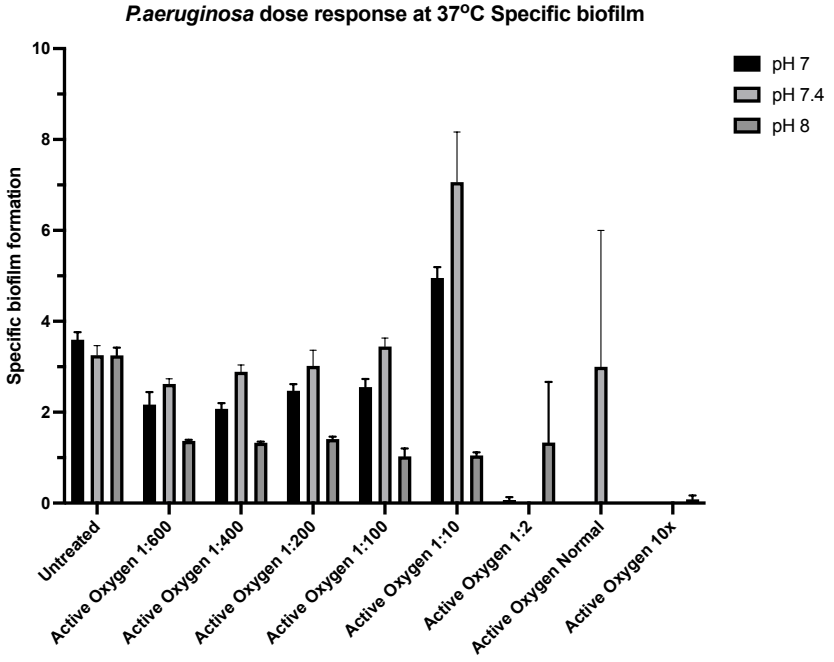


B

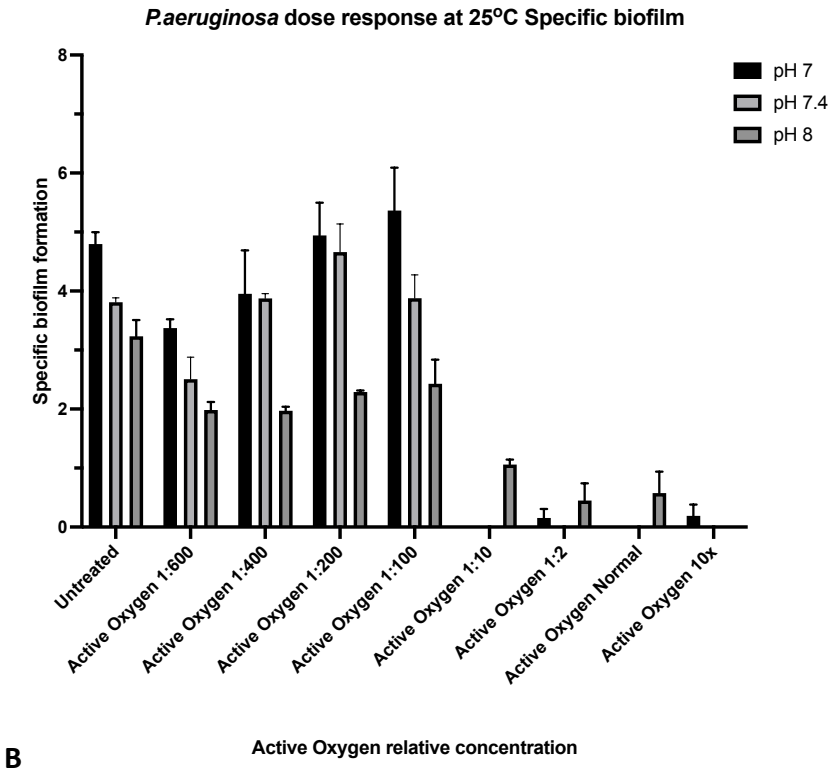
Figures 4.5 A and B show the levels of *E. coli K12* specific biofilm formation in minimal media (M9) and three pH, at two different incubation temperatures, in the presence and absence of various Active Oxygen dilutions. N=3 for each dilution with the exception of the control well (N=4) and the error bars indicate standard error of the mean (SEM).

Specific, or normalized, biofilm formation is used to measure the biofilm-forming ability of an organism per amount of biomass present in the assay (see section 2.7.1). Specific biofilm formation is defined as the absolute biofilm formation (as measured by the level of crystal violet via OD595 readings) divided by the planktonic cell density (measured directly via OD595 per well) to determine the specific biofilm formation, which is without units.

The results from sections 4.2.1-4.2.2 were used to compute specific biofilm formation after treatments with AO at temperatures and pH simulating hot tub environments. As *E. coli* absolute biofilm formation levels were quite low, the specific biofilm formation values generated were also low as a result, and the low OD and absolute biofilm formation values resulted in abnormal spikes at the higher concentration values (Fig. 4.5).



A



B

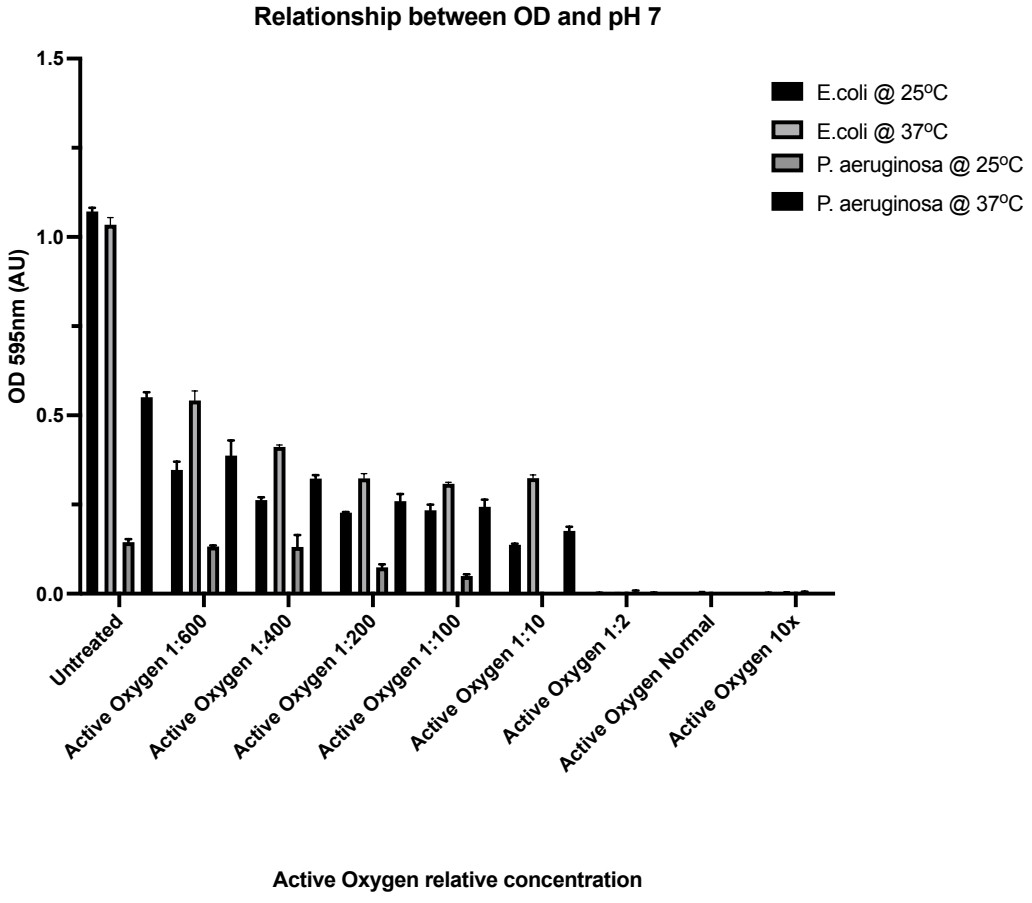
Figure 4.6 A-B Specific biofilm formation of *P. aeruginosa PA01* grown in minimal media (M9) at three pHs, at 37°C (A) and 25°C (B) in the presence and absence of a range of Active Oxygen concentrations. . 'Normal' indicates usage of active oxygen at SOP, other concentrations as indicated relate to normal SOP. N=3 for each data point with the exception of the control (N=4). Error bars indicate standard error of the mean (SEM).

As *P. aeruginosa* is an organism that develops biofilm throughout the well instead of the liquid-air interface as *E. coli* does, absolute biofilm formation tends to be higher. Therefore, specific biofilm formation could potentially provide more useful data regarding the biofilm forming properties of *P. aeruginosa* in response to pH and active oxygen (Fig 4.6).

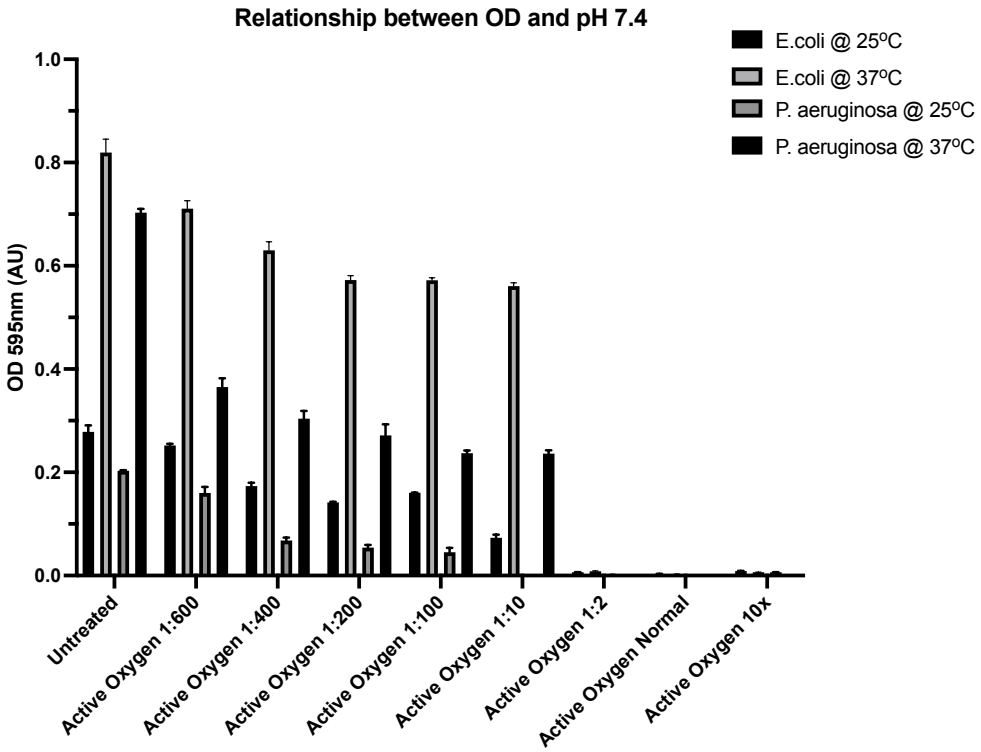
At 37°C at concentrations of half strength of active oxygen and higher, very little specific biofilm can be observed (Fig 4.6A). Peaks in specific biofilm formation can be seen at the one-tenth concentration of active oxygen for *P. aeruginosa* grown at pH 7 and 7.4. These peaks can be attributed to the low planktonic growth values measured. Other than those peaks, the highest levels of specific biofilm formation were observed to be in the control groups, which would be expected. At 25°C at concentrations of half strength of active oxygen and higher little specific biofilm formation is observed (Fig 4.6B). Similar peaks in specific biofilm

formation were observed though peaks seemed to have shifted to lower concentrations. No data points were added for pH7 and 7.4, as the very low numbers of biofilm noted led to the generation of abnormally high numbers. However, large error bars at these concentrations indicate that they may not be of significance. Therefore, the highest specific biofilm formation was observed to be in the control groups, similar to the results at 37^{o}C.

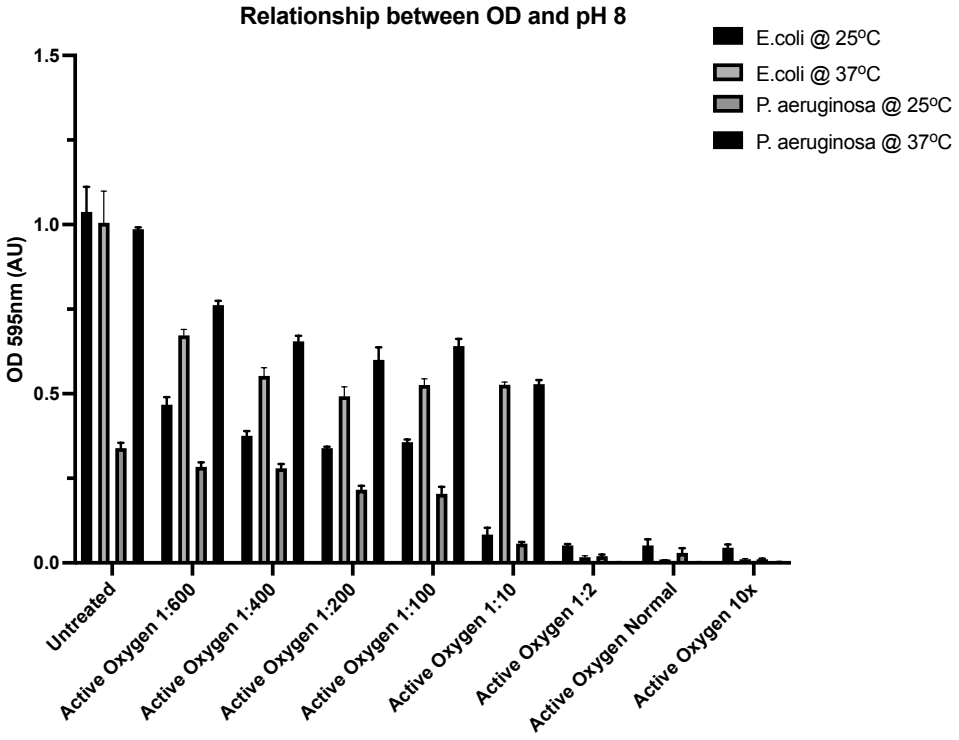
4.2.4 Effect of pH and temperature on efficacy of active oxygen to prevent bacterial growth



A


Relationship between OD and pH 7.4
E.coli @ 25ºC
E.coli @ 37ºC
P. aeruginosa @ 25ºC
P. aeruginosa @ 37ºC
OD 595nm (AU)
1.0
0.8
0.6
0.4
0.2
0.0
Untreated
Active Oxygen 1:600
Active Oxygen 1:400
Active Oxygen 1:200
Active Oxygen 1:100
Active Oxygen 1:10
Active Oxygen 1:2
Active Oxygen Normal
Active Oxygen 10x
Active Oxygen relative concentration

B


Relationship between OD and pH 8
E.coli @ 25ºC
E.coli @ 37ºC
P. aeruginosa @ 25ºC
P. aeruginosa @ 37ºC
OD 595nm (AU)
1.5
1.0
0.5
0.0
Untreated
Active Oxygen 1:600
Active Oxygen 1:400
Active Oxygen 1:200
Active Oxygen 1:100
Active Oxygen 1:10
Active Oxygen 1:2
Active Oxygen Normal
Active Oxygen 10x
Active Oxygen relative concentration

C

Figure 4.7 A-C Planktonic growth of *E. coli K12 and P. aeruginosa PA01* (indicated as OD595) in minimal media (M9) at 37 and 25°C at pH 7 (A), 7.4 (B) and 8 (C) in the presence and absence of a range of Active Oxygen concentrations. 'Normal' indicates usage of active oxygen at SOP, other concentrations as indicated relate to normal SOP. N=3 for each data point with the exception of the control (N=4). Error bars indicate standard error of the mean (SEM).

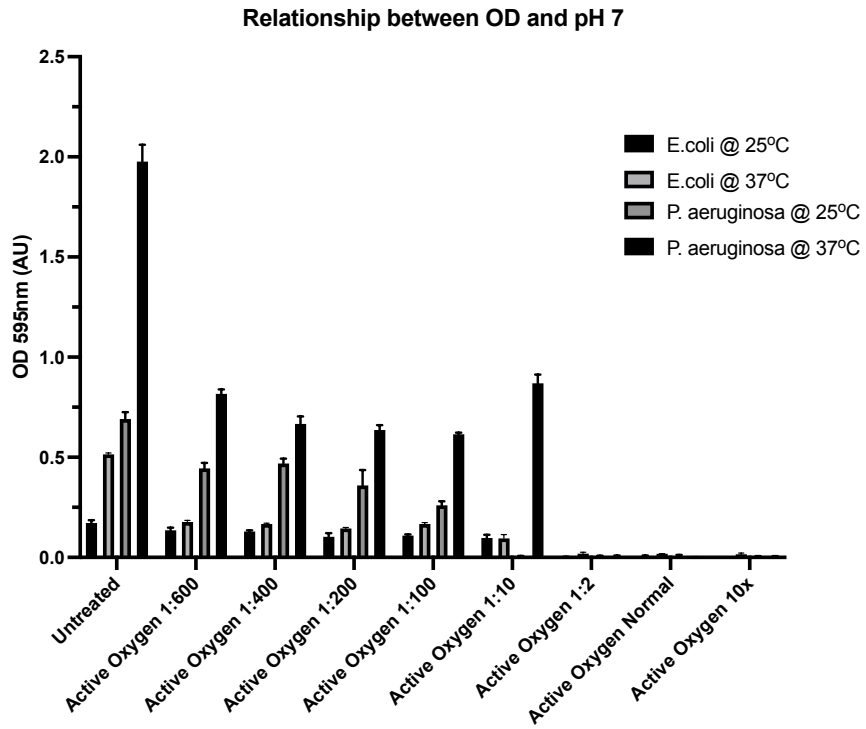
The results were also used to compare the effect of treatments and conditions on planktonic growth of strains at one pH (Fig 4.7 A-C). At pH 7, the highest planktonic growth was exhibited by *E. coli* at both 25 and 37 degrees Celsius, followed by *P. aeruginosa* grown at 37 degrees (Fig 4.7 A). The lowest growth was exhibited by *P. aeruginosa* at 25^{o}C. *E. coli* grown at both temperatures showed a sharp decrease in optical density after treatment with the lowest concentration of Active Oxygen, with *E. coli* grown at 25^{o}C showing a decrease of approximately 67% and *E. coli* at 37^{o}C by approximately 46%. For *P. aeruginosa,* the change is less dramatic, with *P. aeruginosa* grown at 37^{o}C showing a decrease in cell density by approximately 28% and at 25^{o}C by approximately 8.5%. It can be observed that regarding untreated cells, *E. coli* tended to exhibit higher planktonic growth at pH 7 in comparison with *P. aeruginosa.* In addition, *E. coli* appears to be more susceptible to Active Oxygen in comparison with *P. aeruginosa* grown at 37^{o}C. *P. aeruginosa* at 25^{o}C shows a decreased response to Active Oxygen, but also low levels of planktonic growth in comparison with *E. coli*. In addition, a Mann-Whitney U-test between *P. aeruginosa* grown at 37^{o}C and 25^{o}C calculated a significant difference ($P = 0.0286$), while the difference between *E. coli* grown at 37^{o}C and 25^{o}C was calculated as non-significant ($P > 0.05$).

At pH 7.4, *E. coli* grown at 37^{o}C remained at the lead position for highest planktonic growth (Fig 4.7B). However, it is now followed by *P. aeruginosa* grown at 37^{o}C. In contrast with Figure 4.7A, *E. coli* grown at 25^{o}C now showed reduced growth in contrast with the results at pH 7. The overall lowest planktonic growth, similar to pH 7, is shown by *P. aeruginosa* grown at 25^{o}C. In comparison with the results obtained at pH 7, there is an overall decrease in planktonic growth. In conjunction, the overall decrease in planktonic growth can be described as less dramatic. The decrease in planktonic growth in *E. coli* at 37^{o}C is approximately 14%, and for *E. coli* at 25^{o}C it is about 10%. The sharpest decrease in planktonic activity is exhibited by *P. aeruginosa* at 37^{o}C and is about 48%. A decrease of about 21% observed for *P.*

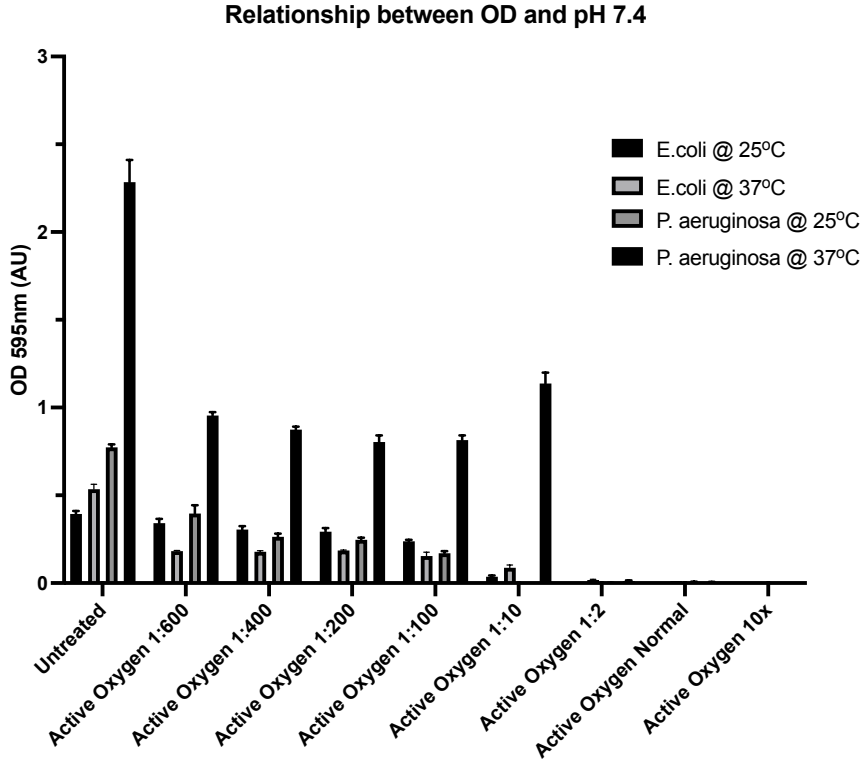
aeruginosa at 25°C. It can be observed that both bacteria grown at 37°C at this pH showed higher planktonic growth than those grown at 25°C. In addition, a Mann-Whitney U-test between *P. aeruginosa* grown at 37°C and 25°C calculated a significant difference (P = 0.0286), which is the same for the difference between *E. coli* at 37°C and 25°C (P = 0.0286). Interestingly, a small peak at media containing 1 in 100 SOP concentration can be observed for *E. coli* and *P. aeruginosa* grown at 37°C.

At pH 8, the highest planktonic growth was exhibited by *E. coli* at 25°C, followed closely by *E. coli* and *P. aeruginosa* grown at 37°C (Fig 4.7C). The lowest planktonic growth was shown by *P. aeruginosa* at 25°C. The sharpest decrease in planktonic activity with the lowest Active Oxygen dilution was observed for *E. coli* grown at 25°C, with a decrease of approximately 53%, and is followed by *E. coli* grown at 37°C with a 33% decrease and *P.* aeruginosa grown at 37°C with a decrease of approximately 21%. The lowest reduction, approximately 11%, was observed for *P. aeruginosa* grown at 25°C. In comparison with the results obtained at pH 7.4, the overall planktonic growth appeared higher. *E. coli* grown at 25°C appeared to be the most responsive organism regarding Active Oxygen treatment at pH 8, while the least responsive one, consistent throughout pH change, appears to be *P. aeruginosa* at 25°C. Interestingly, all bacteria showed a small dip in planktonic growth at the 1:200 SOP concentration, and *E. coli* grown at 37°C showed a small peak at the 1:10 SOP concentration before being quickly reduced. Most importantly, however, some planktonic growth by *E. coli* at 25°C could still be detected, while the very small amounts observed by *E. coli* and *P. aeruginosa* at 37°C are negligible. A Mann-Whitney U-test between *P. aeruginosa* grown at 37°C and 25°C calculated a significant difference (P = 0.0286), while the difference between *E. coli* at 37°C and 25°C was calculated as non-significant (P > 0.05).

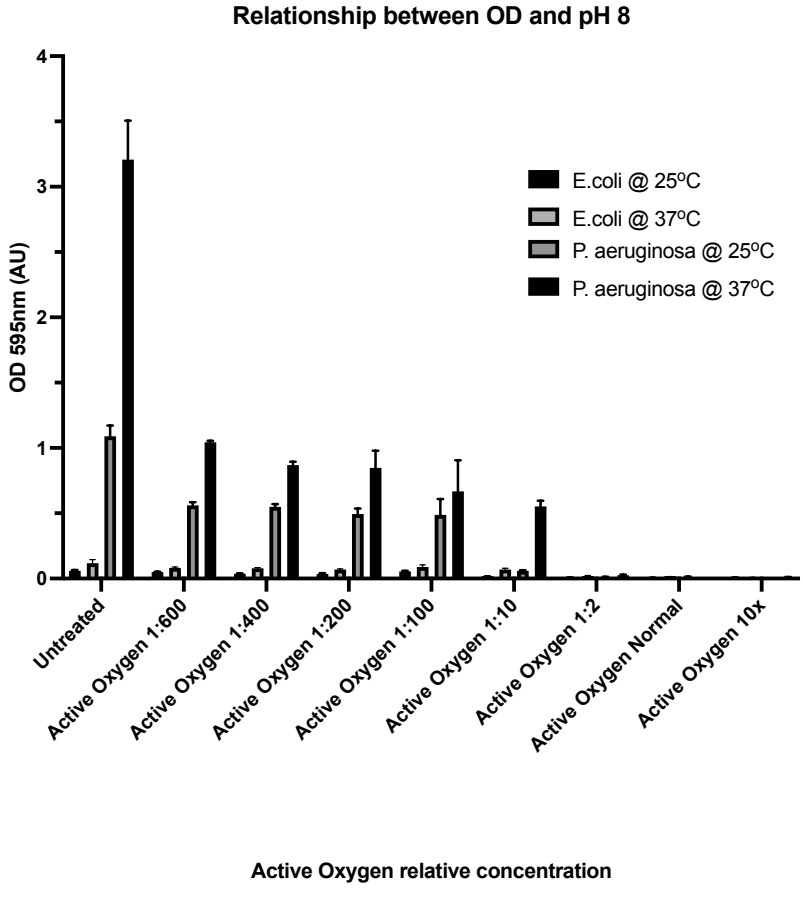
4.2.5 Effect of pH and temperature on efficacy of active oxygen to prevent absolute biofilm formation


Relationship between OD and pH 7
OD 595nm (AU)
2.5
2.0
1.5
1.0
0.5
0.0
E.coli @ 25ºC
E.coli @ 37ºC
P. aeruginosa @ 25ºC
P. aeruginosa @ 37ºC
Untreated
Active Oxygen 1:600
Active Oxygen 1:400
Active Oxygen 1:200
Active Oxygen 1:100
Active Oxygen 1:10
Active Oxygen 1:2
Active Oxygen Normal
Active Oxygen 10x
Active Oxygen relative concentration

A


Relationship between OD and pH 7.4
OD 595nm (AU)
3
2
1
0
E.coli @ 25ºC
E.coli @ 37ºC
P. aeruginosa @ 25ºC
P. aeruginosa @ 37ºC
Untreated
Active Oxygen 1:600
Active Oxygen 1:400
Active Oxygen 1:200
Active Oxygen 1:100
Active Oxygen 1:10
Active Oxygen 1:2
Active Oxygen Normal
Active Oxygen 10x
Active Oxygen relative concentration

B



C

Figure 4.8 Absolute biofilm formation of *E. coli K12* and *P. aeruginosa PA01* (indicated as OD595) grown in minimal media (M9) at 37 and 25°C at pH 7 (A), 7.4 (B) and 8 (C) in the presence and absence of a range of Active Oxygen concentrations. 'Normal' indicates usage of active oxygen at SOP, other concentrations as indicated relate to normal. N=3 for each data point with the exception of the control (N=4). Error bars indicate standard error of the mean (SEM).

The results were also used to compare the effect of treatments and conditions on absolute biofilm formation of strains at one pH (Fig 4.8). At pH 7, the highest biofilm formation in untreated wells was exhibited by *P. aeruginosa* grown at 37ºC, followed by *P. aeruginosa* grown at 25ºC, *E. coli* grown at 37ºC and finally by *E. coli* grown at 25ºC (Fig. 4.8A). *P. aeruginosa* grown at 37ºC showed a remarkable biofilm forming ability, with absolute biofilm endpoint readings measured at ~2.0 AU. Interestingly, *P. aeruginosa* grown at 37ºC also appeared to be very responsive to Active Oxygen, showing a substantial decrease in biofilm

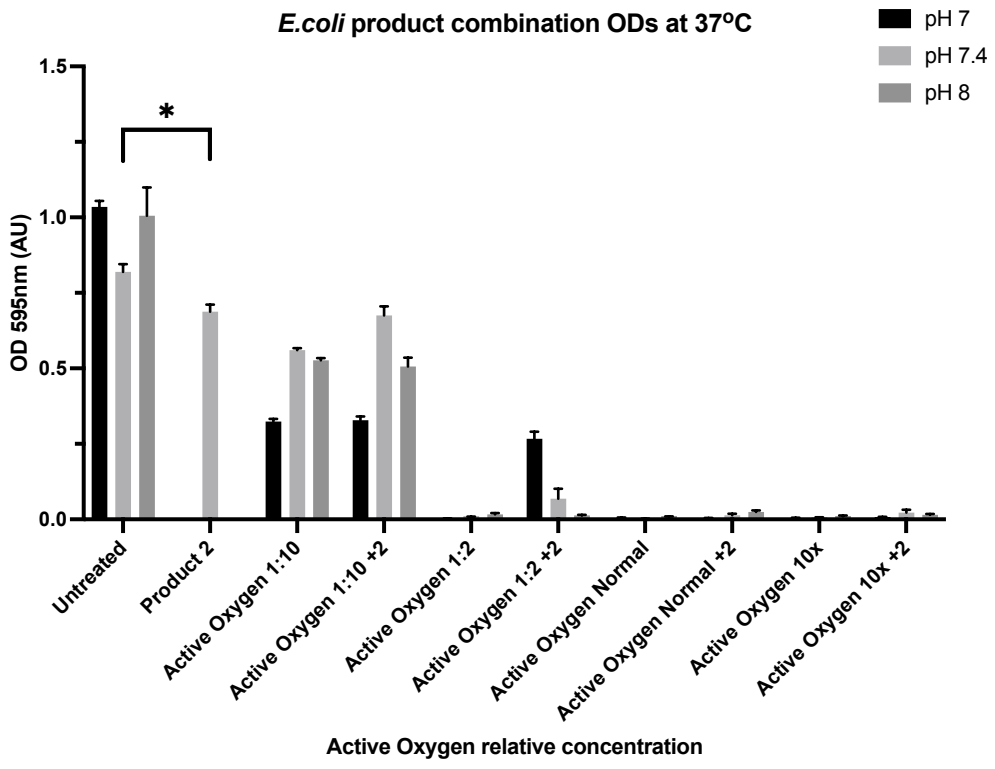
formation after treatment with the lowest concentration of Active Oxygen of approximately 58%. *P. aeruginosa* grown at 25°C showed a decrease of approximately 34%, *E. coli* grown at 37°C of approximately 65%, and *E. coli* grown at 25°C showed the lowest response by approximately 20%. It can be observed that concentrations weaker than the 1 in 10 SOP concentration supported biofilm formation, and active oxygen concentrations of one-tenth or higher were effective at preventing biofilm formation, with almost no biofilm quantified. A small peak in biofilm formation could be observed at one-tenth strength active oxygen for *P. aeruginosa* grown at 37°C, which could be of interest. A Mann-Whitney U-test between *P. aeruginosa* grown at 37°C and 25°C calculated a significant difference (P = 0.0286), which was the same for the difference between *E. coli* grown at 37°C and 25°C (P = 0.0286). Overall, Active Oxygen in concentrations higher than one-tenth strength appeared to be effective against biofilm formation across all strains and temperatures.

At pH 7.4, *P. aeruginosa* grown at 37°C showed the highest biofilm formation across all strains, even higher than at pH 7 and interestingly the dose-response curve follows a trend quite similar to the one at pH 7 (Fig 4.8 B). *P. aeruginosa* grown at 25°C takes the second place, followed closely by *E. coli* grown at both 37 and 25°C. Untreated *E. coli* at 25°C showed higher biofilm formation at pH 7.4 in comparison with pH 7, while *E. coli* at 37°C biofilm formation remained at similar values. *P. aeruginosa* grown at 37°C remained very responsive to Active Oxygen treatment, showing a reduction of approximately 58%, which was the same for pH 7. *P. aeruginosa* grown at 25°C showed a reduction of approximately of 48%, *E. coli* grown at 37°C of approximately 65% and *E. coli* grown at 25°C showed the lowest reduction of about 12%. It can once again be observed that active oxygen concentrations weaker than 1 in 10 SOP support biofilm formation, and dilutions stronger than 1 in 10 SOP are effective at preventing biofilm formation, with very little biofilm quantified. Interestingly, a small peak in biofilm formation can be observed again in the 1 in 10 concentration for *P. aeruginosa* grown at 37°C, which further provokes interest. A Mann-Whitney U-test between *P. aeruginosa* grown at 37°C and 25°C calculated a significant difference (P = 0.0286), which is the same for the difference between *E. coli* grown at 37°C and 25°C (P = 0.0286).

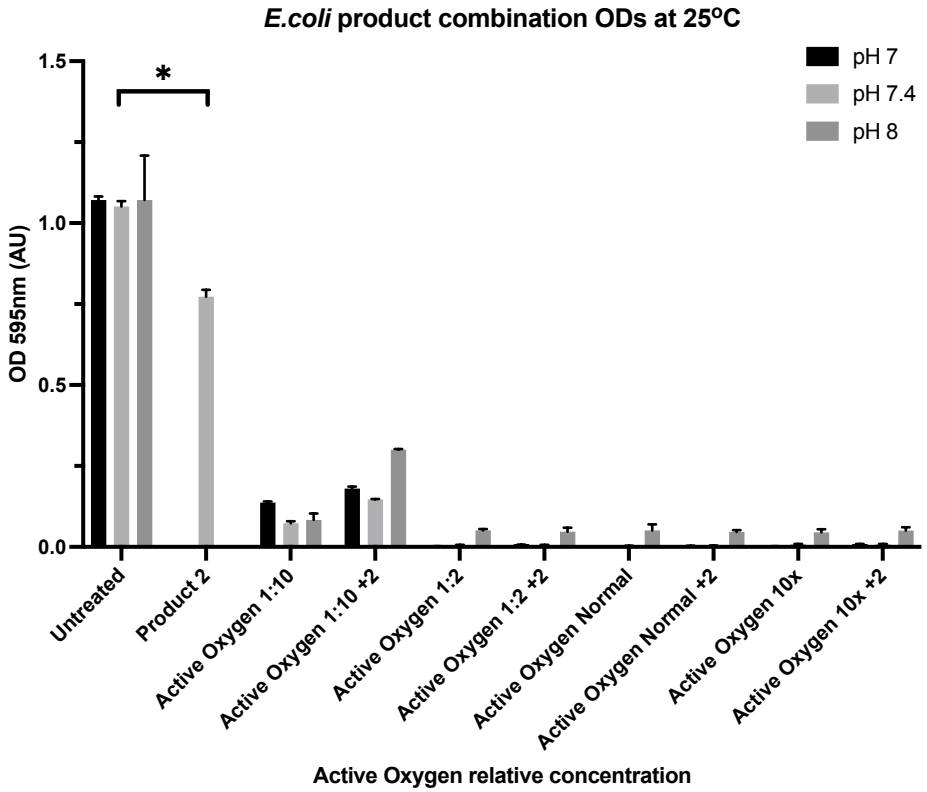
At pH 8, once more the highest biofilm formation was exhibited by *P. aeruginosa* grown at 37°C, which was now at even higher levels than previously reported, with absolute biofilm

endpoint readings measured at > 3.0 AU (Fig 4.8C). The second place is taken by *P. aeruginosa* grown at 25°C, which showed absolute biofilm endpoint readings at ~ 1.1 AU. *E. coli* grown at both temperatures showed little biofilm formation. Once more, *P. aeruginosa* at 37°C remained very responsive to Active Oxygen treatment, showing a reduction of approximately 67%. *P. aeruginosa* grown at 25°C showed a biofilm reduction of approximately of 48%, similar to the reduction noted at pH 7.4, *E. coli* grown at 37°C of approximately 30% and *E. coli* grown at 25°C showed the lowest reduction of about 16%. The peak in biofilm formation shown by *P. aeruginosa* grown at 37°C at one-tength strength active oxygen was no longer present at pH 8. It could once again be observed that concentrations weaker than 1 in 10 SOP supported biofilm formation, and concentrations higher than 1 in 10 were effective at preventing biofilm formation, with miniscule amounts of biofilm quantified. *E. coli* grown at both temperatures showed little biofilm formation at high pH. A Mann-Whitney U-test between *P. aeruginosa* grown at 37°C and 25°C calculated a significant difference (P = 0.0286), which was the same for the difference between *E. coli* grown at 37°C and 25°C (P = 0.0286).

4.2.6 Effect of water conditioner (Product 2) in combination with active oxygen for prevention of planktonic growth in minimal media (M9)


E.coli product combination ODs at 37°C
pH 7
pH 7.4
pH 8
OD 595nm (AU)
1.5
1.0
0.5
0.0
*
Untreated
Product 2
Active Oxygen 1:10
Active Oxygen 1:10 +2
Active Oxygen 1:2
Active Oxygen 1:2 +2
Active Oxygen Normal
Active Oxygen Normal +2
Active Oxygen 10x
Active Oxygen 10x +2
Active Oxygen relative concentration

A


E.coli product combination ODs at 25°C
pH 7
pH 7.4
pH 8
OD 595nm (AU)
1.5
1.0
0.5
0.0
*
Untreated
Product 2
Active Oxygen 1:10
Active Oxygen 1:10 +2
Active Oxygen 1:2
Active Oxygen 1:2 +2
Active Oxygen Normal
Active Oxygen Normal +2
Active Oxygen 10x
Active Oxygen 10x +2
Active Oxygen relative concentration

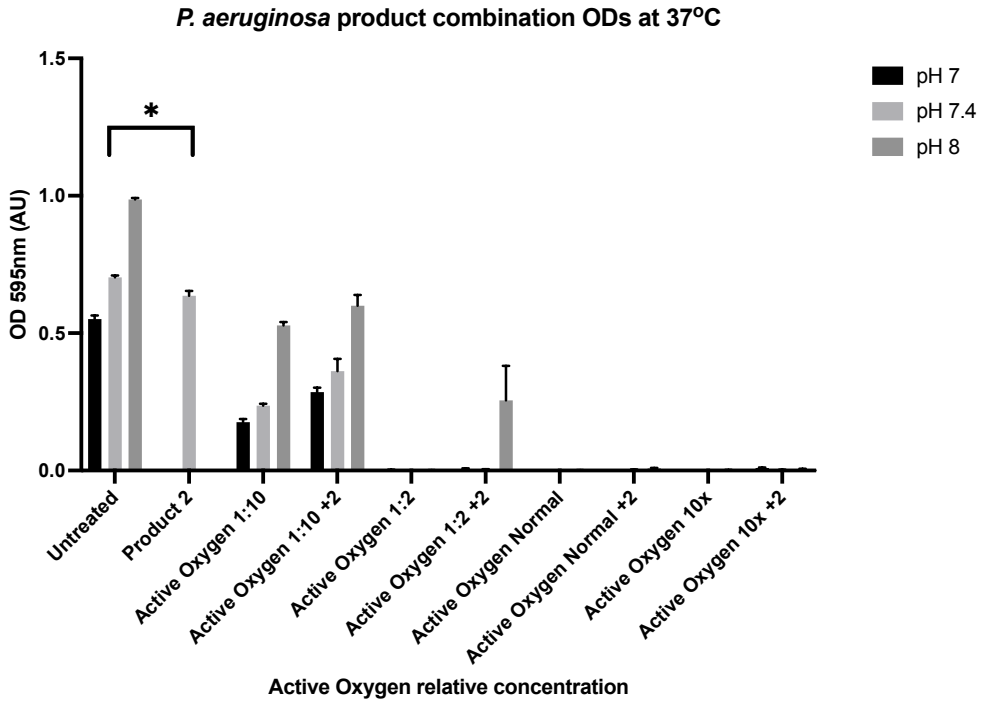
B

Figure 4.9 A and B show the planktonic growth of *E. coli K12* (indicated as OD595) in minimal media (M9) at 37°C (A) and 25°C (B) at pH 7, 7.4 and 8 in the presence and absence of a range of Active Oxygen concentrations and product 2. 'Normal' indicates usage of active oxygen at SOP, other concentrations as indicated relate to normal SOP. Product 2 was used at SOP concentration only. Product 2 alone was tested at pH 7.4 only. N=3 for each data point with the exception of the control (N=4). Error bars indicate standard error of the mean (SEM).

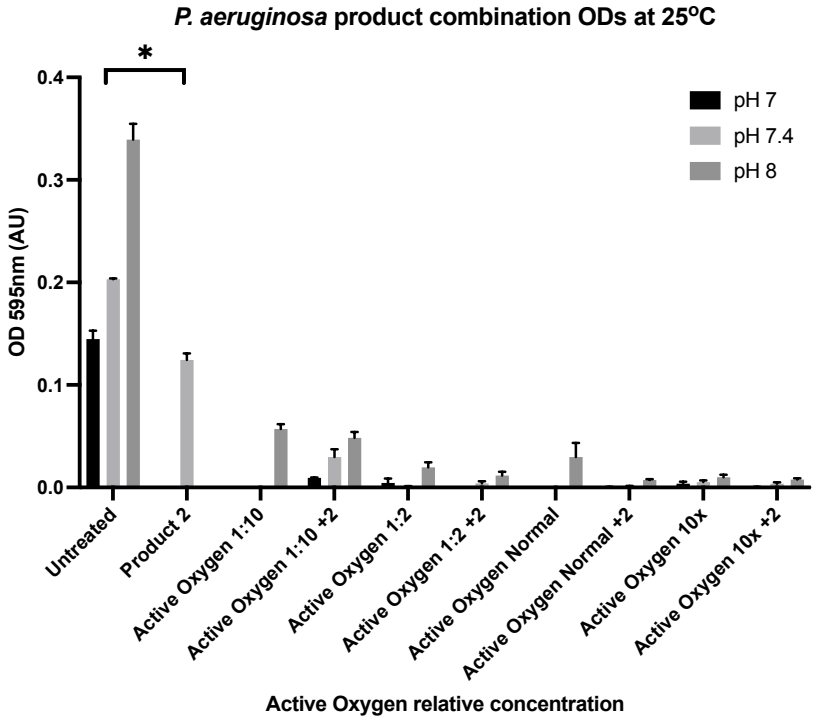
In order to measure the effect of Active Oxygen in combination with Product 2 on planktonic growth, a series of experiments including the SOP concentration as well as weaker and stronger concentrations, added at the time of inoculation at three different pH (7, 7.4 and 8) and two temperatures (25°C and 37°C) were performed. The sponsoring company was in agreement with this. Regarding *E. coli* planktonic growth at 37^{o}C, very little growth was observed at the highest three concentrations of Active Oxygen (1:2, normal and 10x) (Fig 4.9A) The addition of Product 2 by itself appeared to have a small yet significant effect in reducing planktonic growth, confirmed by a Mann-Whitney U-test (P = 0.0286). Notably, the highest planktonic growth excluding controls is observed in the 1 in 10 SOP concentration with the addition of Product 2, with endpoint readings at around 0.56 AU. The addition of Product 2 to active oxygen at one-tenth and half-strength concentrations appeared to enhance planktonic growth when compared to active oxygen at those concentrations alone. Addition of product 2 to active oxygen at higher concentrations did not have an effect. A Scheirer-Ray-Hare test across all combinations showed that Active Oxygen had a significant effect, but that neither Product 2 nor the interaction between the two factors had a significant effect (Critical value for all samples tested <3.85, P > 0.05). A note must be made on the combination of one-tenth strength active oxygen in combination with Product 2, where the critical value was calculated close to the detection limit (approx.3.69), and therefore further investigation and higher replicate numbers may show a combined effect.

Regarding *E. coli* planktonic growth at 25^{o}C, similar to the results at 37^{o}C, very little growth is observed in the higher Active Oxygen concentrations (1:2, normal and 10x) (Fig 4.9B). However, some noticeable growth was observed across all AO concentrations at pH 8. The addition of Product 2 by itself appears to have a small yet significant effect in reducing planktonic growth, confirmed by a Mann-Whitney U-test (P = 0.0286). Product 2 in

combination with the 1 in 10 SOP concentration appears to enhance planktonic, which a Scheirer-Ray-Hare analysis did not calculate as significant. A Scheirer-Ray-Hare test across all combinations showed that Active Oxygen had a significant effect, but that neither Product 2 nor the interaction between the two factors had a significant effect (Critical value for all samples tested <3.85, P > 0.05).



A



B

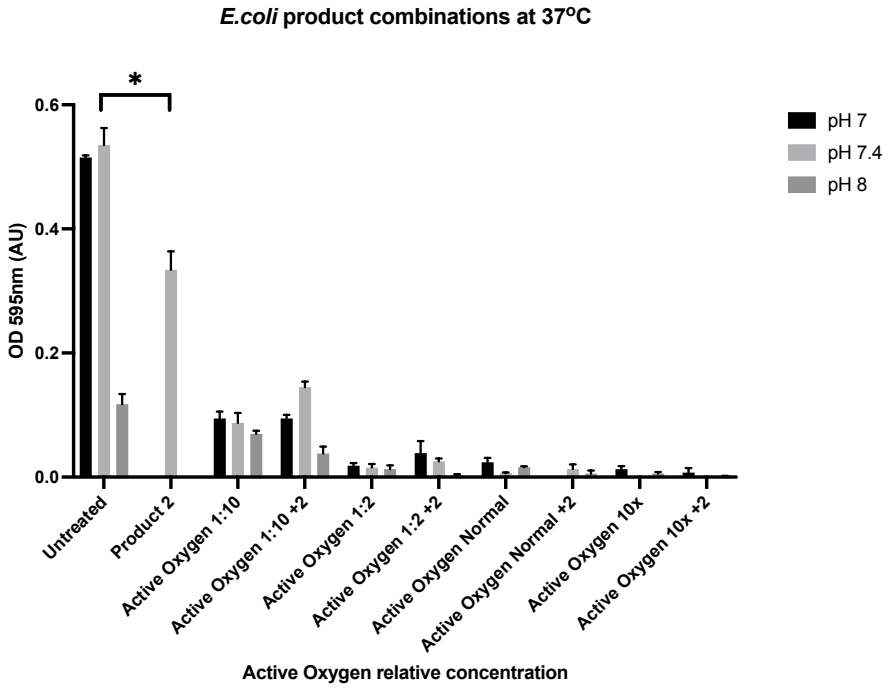
Figures 4.10 A-B Planktonic growth of *P. aeruginosa PA01* (indicated as OD595) in minimal media (M9) at 37°C (A) and 25°C (B) at pH 7, 7.4 and 8 in the presence and absence of a range of Active Oxygen concentrations and product 2. 'Normal' indicates usage of active oxygen at SOP, other concentrations as indicated relate to normal SOP. Product 2 was used at SOP concentration only. Product 2 alone was tested at pH 7.4 only. N=3 for each data point with the exception of the control (N=4). Error bars indicate standard error of the mean (SEM).

By observing the results for *P. aeruginosa* planktonic growth at 37°C (Fig. 4.10 A), very little growth is observed in the higher Active Oxygen concentrations (1:2, normal and 10x). The addition of Product 2 by itself appeared to have a small yet significant effect in reducing planktonic growth, confirmed by a Mann-Whitney U-test ($P = 0.0286$). Notably, the highest levels of planktonic growth is observed in media containing active oxygen of one-tenth dilution, with endpoint readings at around 0.700 AU. A spike in planktonic growth appears in the 1:2 dilution in combination with Product 2 at pH 8, however the large error bars indicate that this is probably of no significance. Product 2 in combination with the one-tenth and 1 in 2 active oxygen concentrations appears to support planktonic growth. The SOP and 10x concentrated preparations in combination with Product 2 had no significant effect on planktonic growth. A Scheirer-Ray-Hare test across all combinations showed that Active Oxygen had a significant effect, but neither Product 2 nor the interaction between the two factors had a significant effect (Critical value for all samples tested <3.85, $P > 0.05$).

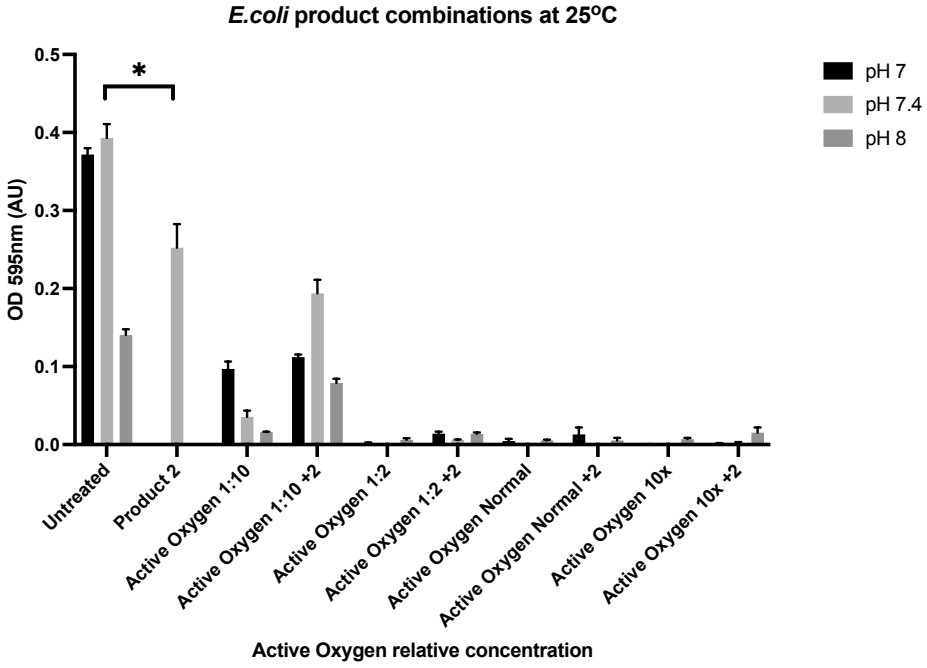
By observing the results for *P. aeruginosa* planktonic growth at 25°C (Fig. 4.10 B), low levels of planktonic growth were supported in media containing active oxygen of 1 in 2 SOP concentration and higher. The addition of Product 2 by itself appears to have a small yet significant effect in preventing planktonic growth, confirmed by a Mann-Whitney U-test ($P = 0.0286$). In comparison with *P. aeruginosa* growth at 37°C (Fig. 4.10 A), Active Oxygen appears less effective at preventing planktonic growth, especially at pH 8. Notably, the highest planktonic growth is observed in the 1 in 10 concentration, with endpoint readings at around 0.06 AU. The addition of Product 2 in media containing active oxygen in the 1 in 10 and 1 in 2 SOP concentrations does not appear to enhance planktonic growth, and a Scheirer-Ray-Hare test across all combinations showed that Active Oxygen had a significant effect but neither

Product 2 nor the interaction between the two factors had a significant effect (Critical value for all samples tested <3.85).

4.2.7 Effect of water conditioner (product 2) on efficacy of active oxygen to prevent absolute biofilm formation



A



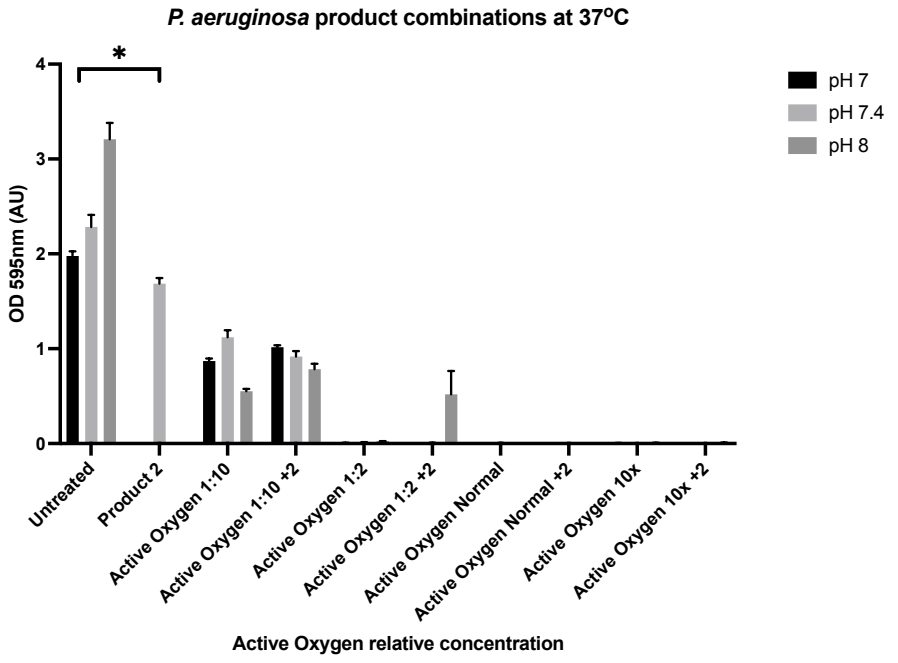
B

Figures 4.11 A-B Absolute biofilm formation of *E. coli K12* (indicated as OD595) grown in minimal media (M9) at 37°C (A) and 25°C (B) at pH 7, 7.4 and 8 in the presence and absence of a range of Active Oxygen concentrations and product 2. 'Normal' indicates usage of active oxygen at SOP, other concentrations as indicated relate to normal SOP. Product 2 was used at SOP concentration only. Product 2 alone was tested at pH 7.4 only. N=3 for each data point with the exception of the control (N=4). Error bars indicate standard error of the mean (SEM).

In order to measure the effect of Active Oxygen in combination with Product 2 on biofilm formation, a series of experiments including the SOP concentration as well as weaker and stronger concentrations, added at the time of inoculation at three different pH (7, 7.4 and 8) and two temperatures (25°C and 37°C) were performed. The sponsoring company was in agreement with this. The exact values of pH used were chosen as the activity of active oxygen under different pH has been demonstrated to remain stable, in comparison with chlorine at the same values (Chapter 3). Regarding *E. coli* absolute biofilm formation at 37°C (Fig. 4.11 A), small amounts of biofilm were supported across all Active Oxygen concentrations. The addition of Product 2 by itself appeared to have a small yet significant effect in reducing biofilm formation, confirmed by a Mann-Whitney U-test ($P = 0.0286$). The highest biofilm formation in treated wells is observed in media containing active oxygen in the 1 in 10 concentration in combination with Product 2, with endpoint readings at around 0.14 AU. A Scheirer-Ray-Hare test across all combinations showed that Active Oxygen had a significant effect but neither Product 2 nor the interaction between the two factors had a significant effect (Critical value for those samples tested <3.85, $P > 0.05$). Small amounts of biofilm could be observed across all concentrations of AO and pH, with the lowest numbers seen at the wells treated with AO at Normal and 10x concentrations.

For *E. coli* absolute biofilm formation at 25°C (Fig. 4.11 B), small amounts of biofilm were observed across all Active Oxygen concentrations. The addition of Product 2 by itself appeared to have a small yet significant effect in preventing biofilm formation, confirmed by a Mann-Whitney U-test ($P = 0.0286$). The highest levels of biofilm formation in treated wells was supported in the media containing 1 in 10 SOP concentration + Product 2, with endpoint readings at around 0.2 AU. The addition of Product 2 in combination with active oxygen does not appear to affect biofilm formation. A Scheirer-Ray-Hare test across all combinations

showed that Active Oxygen had a significant effect but neither Product 2 nor the interaction between the two factors had a significant effect (Critical value for all samples tested <3.85), however the critical value noted for Active Oxygen with the addition for Product 2 was elevated (approx. 2.6), which could be further investigated. Small amounts of biofilm were supported across all concentrations and pHs, with the lowest numbers seen at bacteria treated with the SOP and 10x concentrations.



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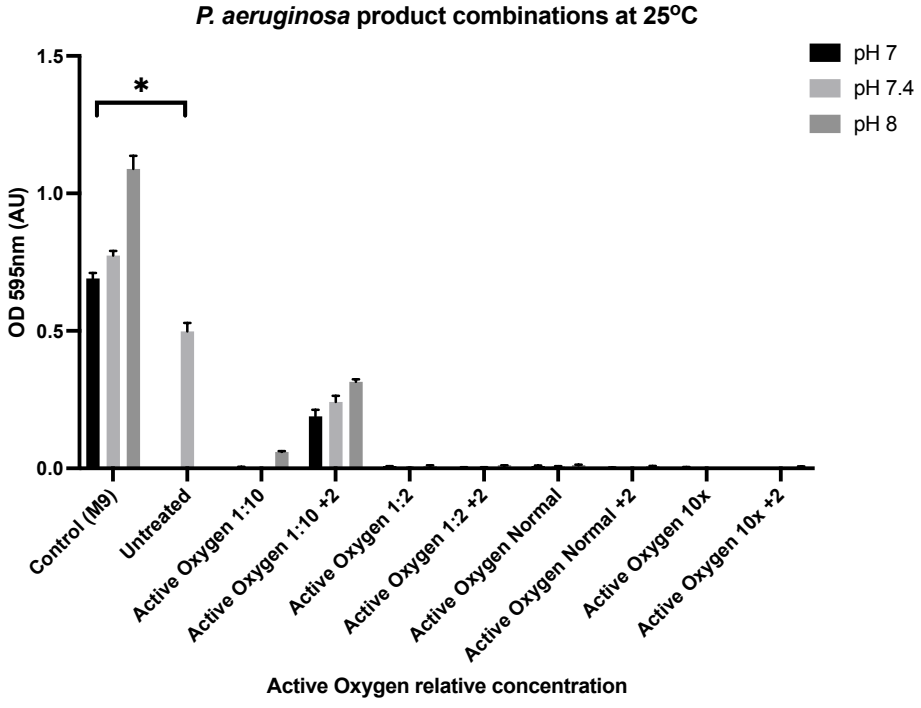


B

Figures 4.12 A-B Absolute biofilm formation of *P. aeruginosa PA01* (indicated as OD595) grown in minimal media (M9) at 37°C (A) and 25°C (B) at pH 7, 7.4 and 8 in the presence and absence of a range of Active Oxygen concentrations and product 2. 'Normal' indicates usage of active oxygen at SOP, other concentrations as indicated relate to normal SOP. Product 2 was used at SOP concentration only. Product 2 alone was tested at pH 7.4 only. N=3 for each data point with the exception of the control (N=4). Error bars indicate standard error of the mean (SEM).

Regarding *P. aeruginosa* absolute biofilm formation at 37^{o}C after Active Oxygen treatment (Fig. 4.12 A), small levels of biofilm were supported in the 1 in 2 SOP concentration with the addition of Product 2 at pH 8, however the error bar is quite large, so it probably was of no significance. The addition of Product 2 by itself appears to have a small yet significant effect in preventing biofilm formation, confirmed by a Mann-Whitney U-test (P = 0.0286). Little to no biofilm was supported in all concentrations and combinations stronger than 1 in 10 SOP concentration, with the exception of the 1 in 2 concentration + Product 2. No biofilm was observed at all in the SOP concentration and 10x concentration, and very small amounts were supported in these concentrations at pH 8. A Scheirer-Ray-Hare test across all combinations showed that Active Oxygen had a significant effect but neither Product 2 nor the interaction between the two factors had a significant effect (Critical value for all samples tested <3.85).

By observing the results of *P. aeruginosa* absolute biofilm formation at 25^{o}C (Fig.4.12 B), small amounts of biofilm were supported across all Active Oxygen concentrations. The addition of Product 2 by itself appears to have a small yet significant effect in preventing biofilm formation, confirmed by a Mann-Whitney U-test (P = 0.0286). Very little biofilm was supported in media with active oxygen concentrations higher than 1in 10, with almost no biofilm observed at all in the 10x SOP concentration. The combination of Active Oxygen in the 1 in 10 SOP concentration with Product 2 lead to low levels of biofilm formation. Small amounts of biofilm can be seen at the SOP and 10x concentration at pH 8. A Scheirer-Ray-Hare test across all combinations showed that Active Oxygen had a significant effect but neither Product 2 nor the interaction between the two factors had a significant effect (Critical value for all samples tested <3.85). However, the combination of Active Oxygen in the 1 in 10

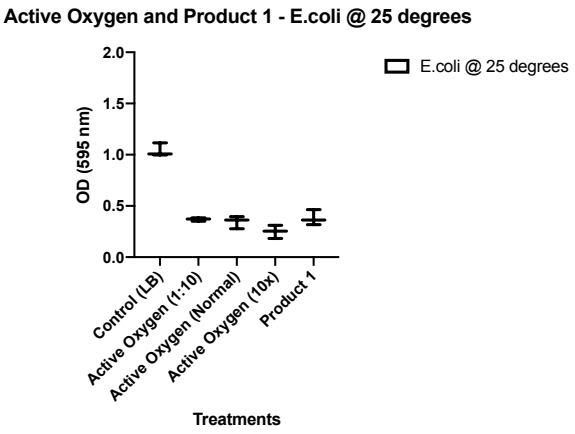
SOP concentration in combination with Product 2 generated an increased critical value (approx. 2.1), which may lead to further investigation.

4.2.8 Static planktonic removal assays in nutrient rich media – Non S.O.P. experiments

The experiments described from this section onwards were all performed before the outbreak of COVID-19 under non-SOP conditions. As the project was at a relatively early stage, many of the experiments described here were experimental and included product combinations not normally found in a hot tub, to assess product interactions and their effect in biofilm inhibition/prevention.

This section contains the results obtained from the static planktonic growth inhibition assays performed on two bacterial species, *E. coli K12* and *P. aeruginosa PA01* at two different temperatures, 25 and 37 degrees Celsius. The results represent cell densities (OD595) obtained after 24hrs of incubation in LB media and a further 24hrs (total 48hrs) incubation after addition of active oxygen at different concentrations and product combinations, including the active oxygen tablets (Product 3), the water conditioner (Product 2) as well as the biofilm remover (Product 1) for *E. coli* (Fig 4.13) and *P. aeruginosa* (Fig. 4.14). Only the final cell densities were recorded and are reported here. Fresh media was added in the control wells and the products added were diluted in water, not media.

It must be made clear that the researcher wished to show the data here, even though they were not optimized to show trends. In addition, due to experimental limitations, statistical analyses were not performed.



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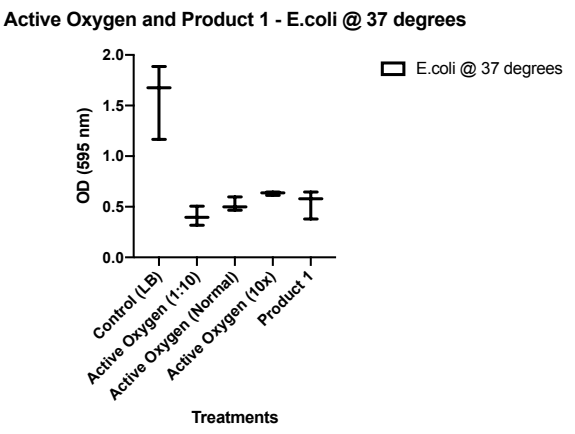
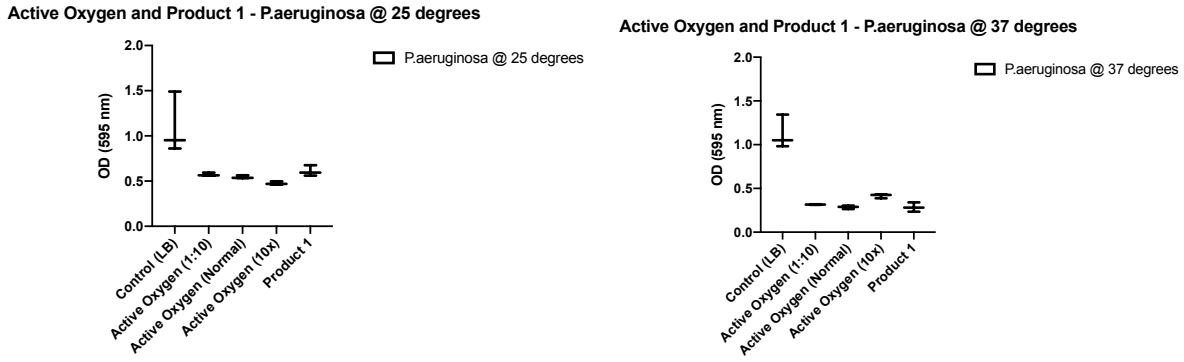


B

Figures 4.13 A and B. Planktonic growth of *E. coli K12* (indicated as OD595) in nutrient-rich media (LB) at 25°C (A) and 37°C (B) in the presence and absence of a range of Active Oxygen concentrations and product 1 (biofilm remover). 'Normal' indicates usage of active oxygen at SOP, other concentrations as indicated relate to normal SOP. Product 1 was used at SOP concentration only. N=3 for each data point. Error bars indicate upper/lower quartile limits.

Untreated *E. coli* at 37°C showed the highest planktonic growth in comparison with *E. coli* at 25°C, with endpoint readings at around 1.5 AU, in comparison to *E. coli* at 25°C 1.0 AU (Fig. 4.13). Active Oxygen appeared to be effective at reducing planktonic growth across all tested concentrations. Product 1 (biofilm remover), used at SOP, also appeared effective at reducing planktonic growth.

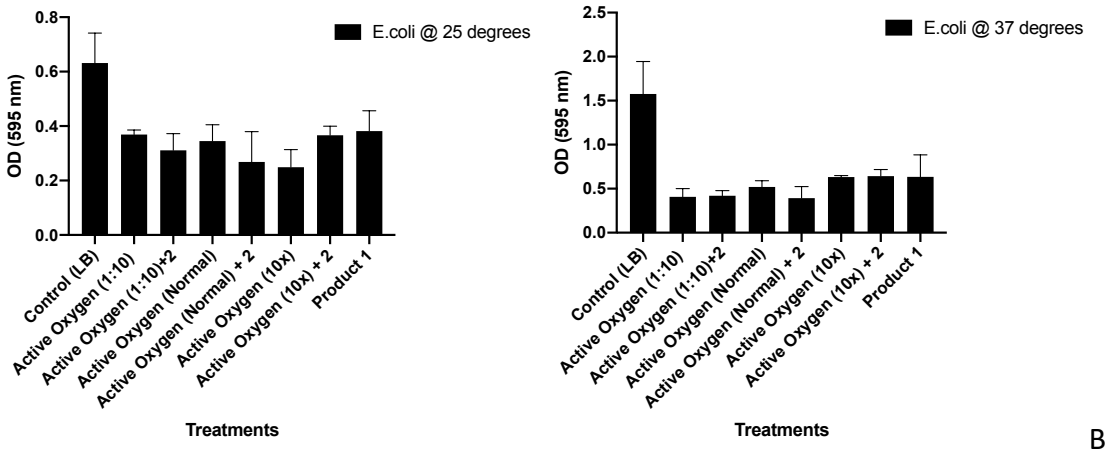


A B

Figure 4.14 A-B. Planktonic growth of *P. aeruginosa PA01* (indicated as OD595) in nutrient-rich media (LB) at 25°C (A) and 37°C (B) in the presence and absence of a range of Active Oxygen concentrations and product 1 (biofilm remover). 'Normal' indicates usage of active oxygen at SOP, other concentrations as indicated relate to normal SOP. Product 1 was used at SOP concentration only. N=3 for each data point. Error bars indicate upper/lower quartile limits.

Untreated *P. aeruginosa* at 37°C showed the highest planktonic growth in comparison with *P. aeruginosa* at 25°C, with endpoint readings at around 1.12 AU, in comparison to *P. aeruginosa* at 25°C 1.10 AU (Fig. 4.14). Active Oxygen appeared to be effective at reducing planktonic

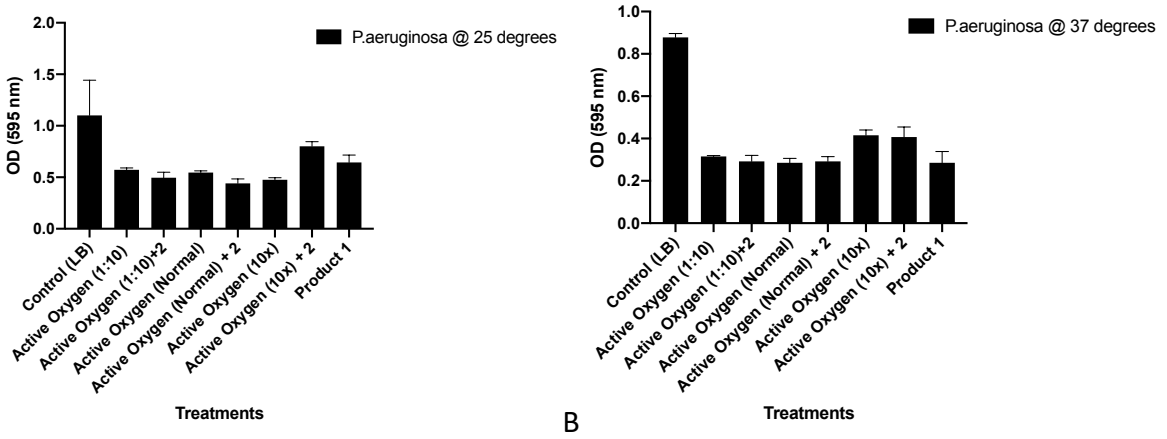
growth across all concentrations, especially at 37^{o}C. Product 1 (biofilm remover) also appeared effective at reducing planktonic growth.



A B

Figures 4.15 Planktonic growth of *E. coli K12* (indicated as OD 595) in nutrient-rich media (LB) at 25°C (A) and 37°C (B) in the presence and absence of a range of Active Oxygen concentrations, plus with Products 1 by itself (biofilm remover) and active oxygen in combination with Product 2 (water conditioner). Product 2 was not used by itself. 'Normal' indicates usage of active oxygen at SOP, other concentrations as indicated relate to normal SOP. Product 1 was used at SOP concentration only. N=3 for each data point. Error bars indicate standard error of the mean (SEM).

The aim of this experiment was to assess the planktonic growth inhibitory effect of active oxygen against *E. coli* and *P. aeruginosa* in various concentrations by itself as well as in combination with product 2, as well as the effect of Product 1, even though it is marketed as a biofilm remover. Regarding the cell density measurements of *E. coli* at both temperatures, it was observed that treating cells with Active Oxygen across all concentrations was effective at reducing planktonic growth by up to 74.28 % (Fig. 4.15 B). The addition of Product 2 to active oxygen treatments did not appear to have a significant effect in growth prevention. In addition, Active oxygen and its combination with Product 2 appeared to be more effective at 37^{o}C.



Figures 4.16 Planktonic growth of *P. aeruginosa* PAO1 (indicated as OD595) in nutrient-rich media (LB) at 25°C (A) and 37°C (B) in the presence and absence of a range of Active Oxygen concentrations, plus with Products 1 (biofilm remover) and in combination with Product 2 (water conditioner). 'Normal' indicates usage of active oxygen at SOP, other concentrations as indicated relate to normal SOP. Product 1 was used at SOP concentration only. N=3 for each data point. Error bars indicate standard error of the mean (SEM).

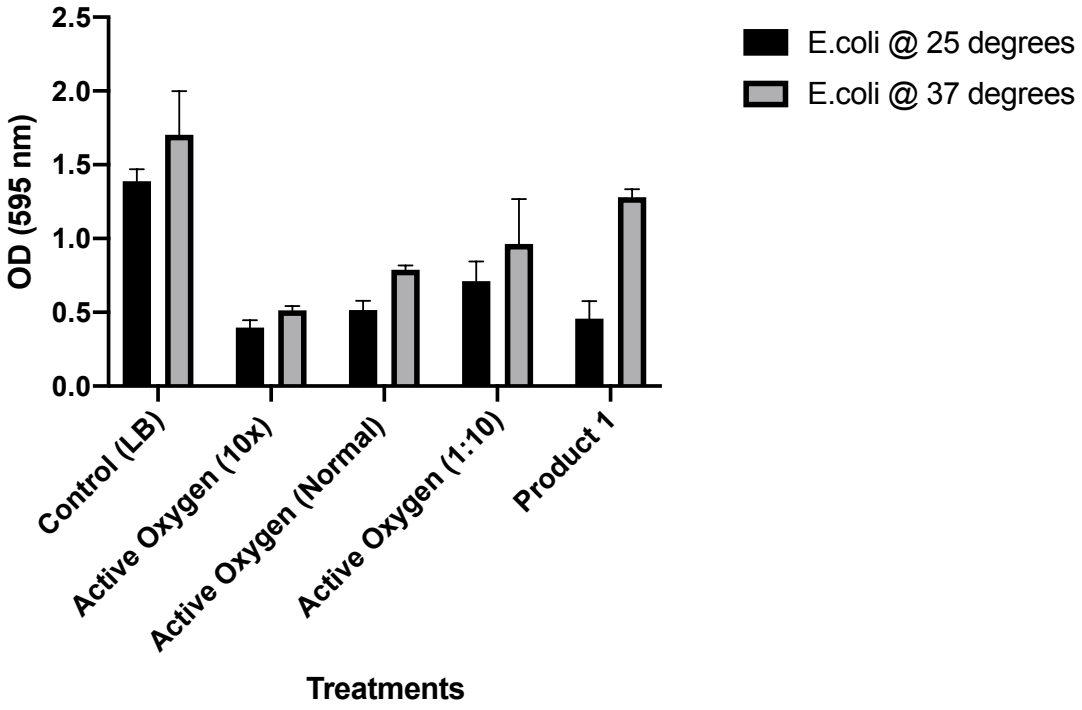
Regarding the cell density measurements of *P. aeruginosa* at both temperatures, it can be observed that Active Oxygen across all concentrations was effective at reducing planktonic growth by up to 67.5% (Fig, 4.16 B), and the addition of Product 2 did not appear to have a significant effect in growth prevention (Fig 4.16 A-B). A small increase in planktonic growth could be observed when active oxygen was added at 10x concentration at both temperatures, however it is possible that this could have been due to accidental contamination and therefore of no significance.

4.2.9 Static biofilm removal in nutrient rich media – Non S.O.P. experiments

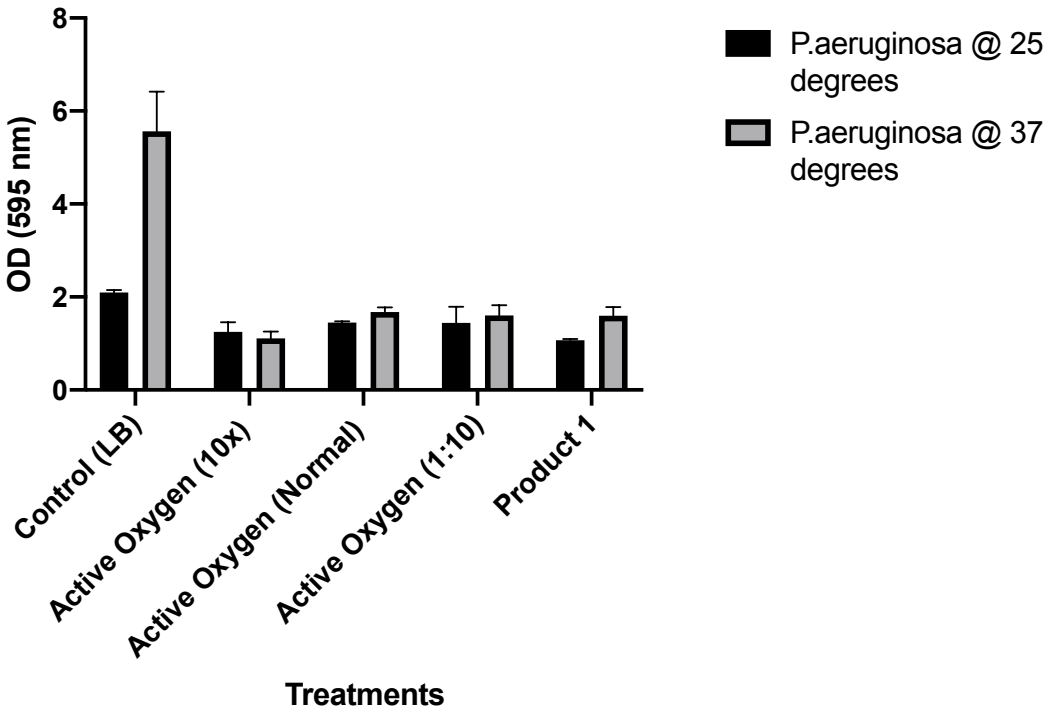
This section contains the results obtained from the static biofilm removal assays performed on *E. coli K12* and *P. aeruginosa PA01* at two different temperatures, 25 and 37 degrees Celsius. The results represent the absolute biofilm formed (measured as OD at 595nm) obtained after 24hrs of incubation in LB media and a further 24hrs (total 48hrs) incubation

with the addition of active oxygen dilutions and/or other Eco3Spa products and combinations, followed by crystal violet staining (measured at OD595) for *E. coli* and *P. aeruginosa* (Fig. 4.17). Only the final cell densities were recorded and are reported here. Fresh media was added in the control wells and the products added were diluted in water, not media.

It must be made clear that the researcher wished to show the data here, even though they were not optimized to show trends. In addition, due to experimental limitations, statistical analyses were not performed.



A



B

Figures 4.17 A-B Absolute biofilm formation (indicated as OD595) of *E. coli K12* (A) and *P. aeruginosa* PAO1 (B) in nutrient-rich media (LB) in the presence and absence of a range of Active Oxygen concentrations or product 1 (biofilm remover), which were added after an initial 24hrs of growth and biofilm formation. 'Normal' indicates usage of active oxygen at SOP, other concentrations as indicated relate to normal SOP. Product 1 was used at SOP concentration only. N=3 for each data point. Error bars indicate standard error of the mean (SEM).

The aim of this experiment was to assess the biofilm removal effect of active oxygen against developed *E. coli* and *P. aeruginosa* biofilms after 24hrs, *in* various concentrations by itself as well as in combination with product 2, as well as the effect of Product 1, even though it is marketed as a biofilm remover. By observing the absolute biofilm formation results for *E. coli* (Fig. 4.17 A) it can be seen that untreated and treated *E. coli* showed higher biofilm formation at 37^{o}C compared to 25^{o}C. Addition of Active Oxygen at all concentrations appeared to be effective at reducing biofilms that were formed after 24hrs and/or prevented or reduced further biofilm formation in comparison to the untreated samples. In addition, Product 1 (biofilm remover) removed biofilms and/or reduced biofilm formation more effectively at 25^{o}C in comparison to 37^{o}C.

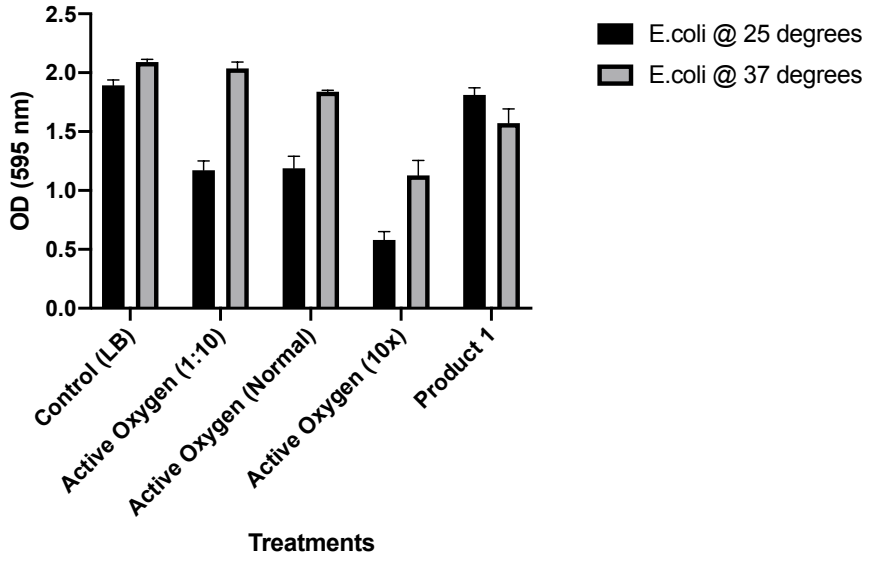
By looking at the absolute biofilm formation results for *P. aeruginosa* (Fig. 4.17 B), it is evident that it retained and/or formed higher biofilm levels at 37^{o}C in comparison to 25^{o}C. *P. aeruginosa* also showed higher biofilm formation in comparison with *E. coli* at both temperatures. Addition of Active Oxygen at all concentrations appeared to be effective at removing biofilms that were formed after 24hrs and/or prevented or reduced further biofilm formation in comparison to the untreated samples, and effectiveness appears to be maintained across different concentrations. In addition, Product 1 removed biofilms and/or reduced biofilm formation at both temperatures.

4.2.10. Static planktonic growth prevention assays in M9 minimal media – Non S.O.P. experiments

This section contains the results obtained from the static biofilm removal assays performed on two bacterial species, *Escherichia coli K12* and *Pseudomonas aeruginosa PA01* at two different temperatures, 25 and 37 degrees Celsius. The results represent the optical densities obtained after 24hrs incubation in M9 minimal media.

Fresh media was added in the control wells and the products added were diluted in water, not media.

It must be made clear that the researcher wished to show the data here, even though they were not optimized to show trends. In addition, due to experimental limitations, statistical analyses were not logical and were not performed.



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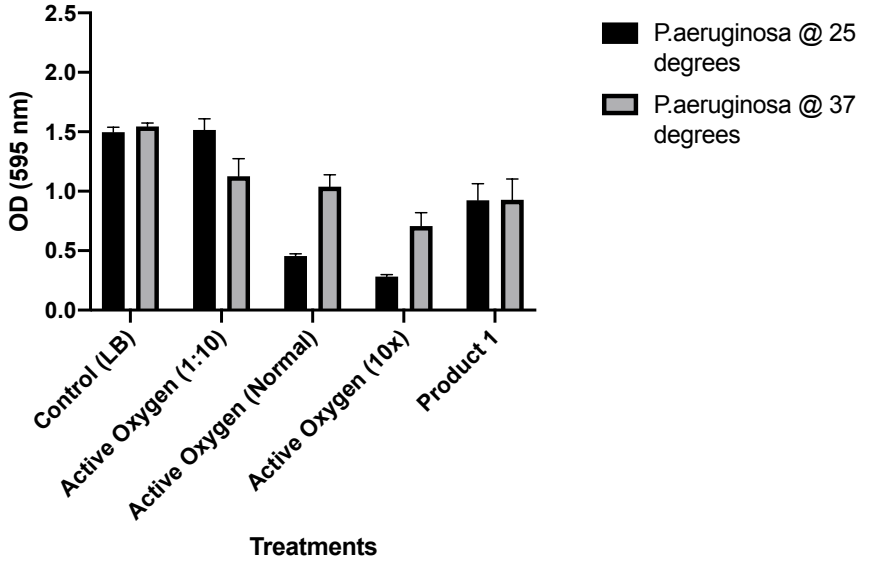


B

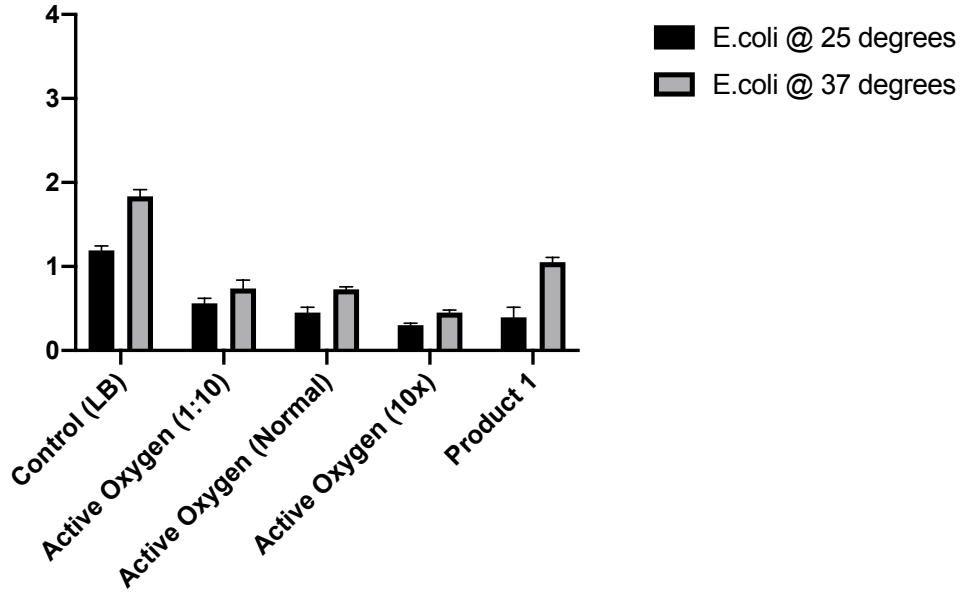
Figures 4.18 A and 4.18 B Planktonic growth of *E. coli K12* and *P. aeruginosa* (indicated as OD 595) in minimal media (M9) at 25°C (A) and 37°C (B) in the presence and absence of a range of Active Oxygen concentrations, plus with Products 1 by itself (biofilm remover). 'Normal' indicates usage of active oxygen at SOP, other concentrations as indicated relate to normal SOP. Product 1 was used at SOP concentration only. N=3 for each data point. Error bars indicate standard error of the mean (SEM).

The aim of this experiment was to assess the planktonic growth inhibitory effect of active oxygen against *E. coli* and *P. aeruginosa* after 24hrs, *in* various concentrations by itself as well as in combination with product 2, as well as the effect of Product 1, even though it is marketed as a biofilm remover. Untreated *E. coli* samples at 37^{o}C (Fig. 4.18 A) show the highest planktonic growth with an approximate endpoint reading of 2.1 AU, followed by *E. coli* at 25^{o}C with an approximate endpoint reading of 1.9 AU. Active Oxygen across all dilutions appears to have an effect in reducing planktonic growth, with the greatest effect observed in the 10x concentration. Interestingly, the product appears to be more effective at 25^{o}C in comparison to 37^{o}C, where little effect is observed. In addition, Product 1 does not appear to be very effective at preventing biofilm formation at either temperature.

Untreated *P. aeruginosa* samples at 37^{o}C (Fig. 4.18 B) show the highest planktonic growth with an approximate endpoint reading of 1.6 AU, followed by *P. aeruginosa* at 25^{o}C with an approximate endpoint reading of 1.4 AU. Similar to the results for *E. coli* (Fig. 4.18 A), Active Oxygen across all dilutions appears to have an effect in reducing planktonic growth, with the greatest effect observed in the 10x concentration. The only exception appears to be Active Oxygen in 1:10 dilution at 25^{o}C, where almost no effect is observed. In general, the product appears to be more effective at 25^{o}C. In addition, Product 1 appears to have an effect at preventing biofilm formation to a similar degree in both temperatures, in contrast with *E. coli.*

4.2.11 Static biofilm removal assays in minimal media – Non S.O.P. experiments

This section contains the results obtained from the static biofilm removal assays performed on two bacterial species, *Escherichia coli K12* and *Pseudomonas aeruginosa PA01* at two different temperatures, 25 and 37 degrees Celsius. The results represent the absolute biofilm formation (measured as OD at 595nm) obtained after 24hrs of incubation in M9 minimal media and a further 24hrs incubation with the addition of active oxygen dilutions and product combinations, followed by crystal violet staining.



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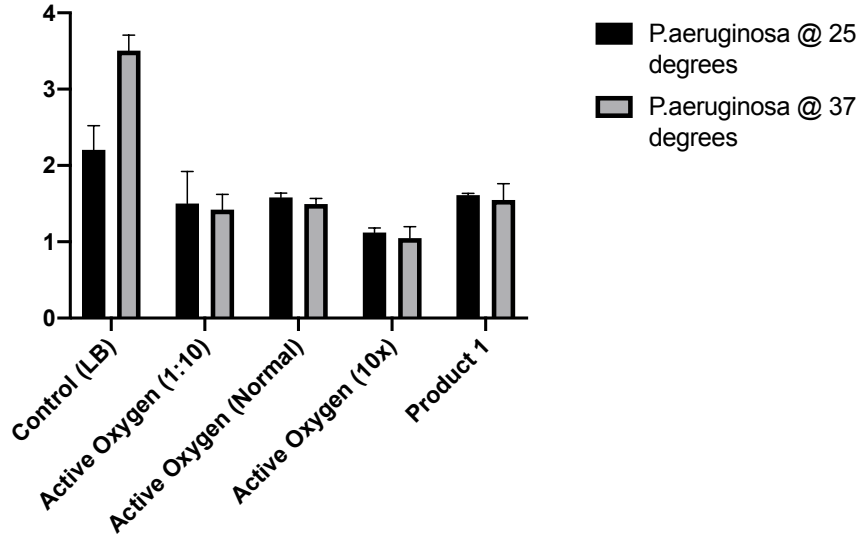


B

Figures 4.19 A and 4.19 B show the biofilm formation measured after performing a crystal violet assay and measuring the absorbance at 595nm (absolute biofilm?) in *E. coli* (A) and *P. aeruginosa* (B), in the presence and absence of the active oxygen product as well as product 1 (biofilm remover). N=3 for each treatment and error bars indicate standard error of the mean.

The aim of this experiment was to assess the biofilm prevention effect of active oxygen against *E. coli* and *P. aeruginosa* after 24hrs, in various concentrations by itself as well as the effect of Product 1, even though it is marketed as a biofilm remover. By observing the absolute biofilm formation results for *E. coli* (Fig. 4.19 A) it can be seen that *E. coli* shows higher biofilm formation at 37^{o}C compared to 25^{o}C. All Active Oxygen concentrations appear to be effective at reducing biofilm formation in comparison to the untreated samples. Interestingly Active Oxygen appears to be more effective at preventing biofilm formation at 25^{o}C compared to 37^{o}C. The greatest preventative effect is observed at the 10x concentration. Product 1 shows a more pronounced effect at 25^{o}C compared to 37^{o}C.

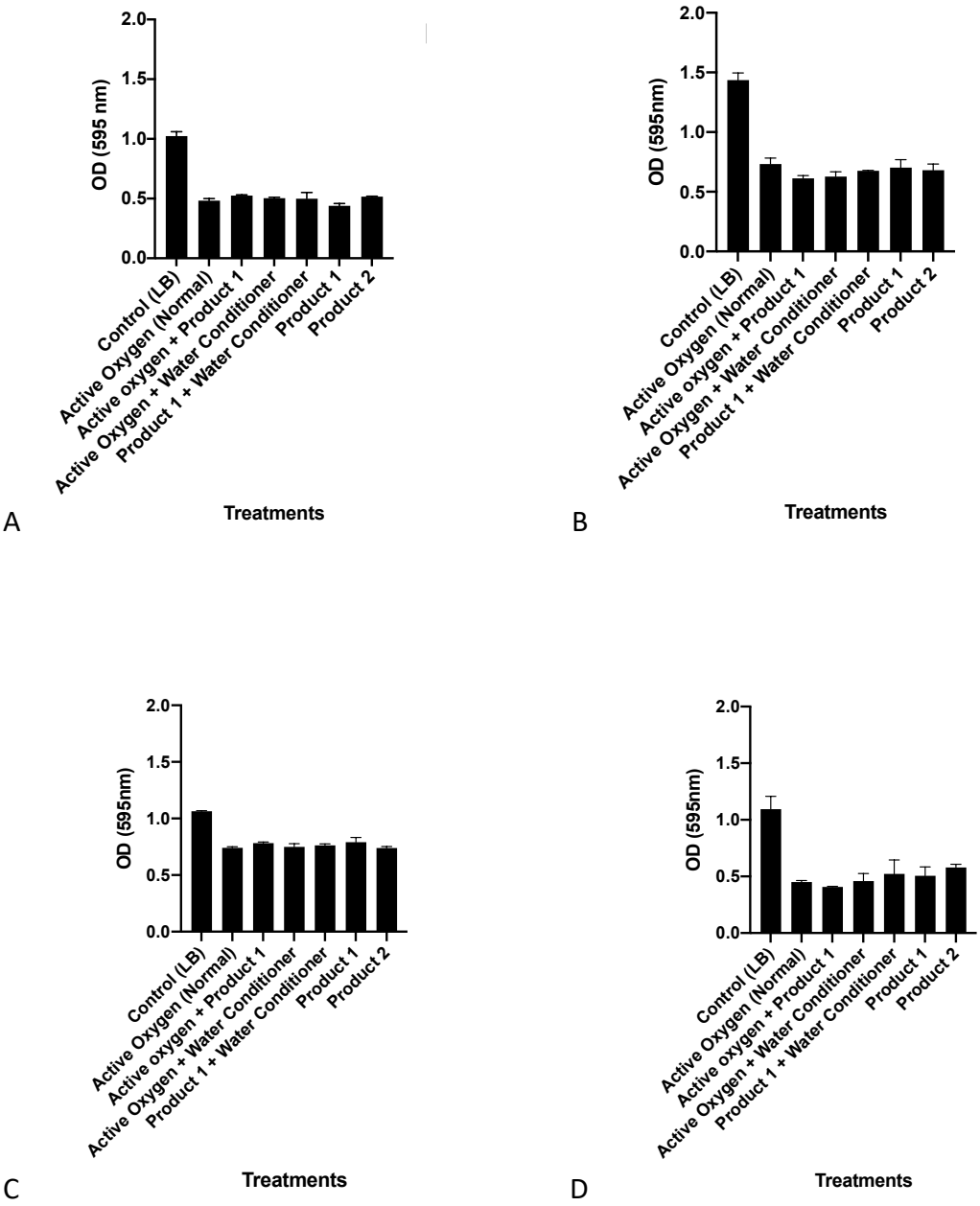
By looking at the absolute biofilm formation results for *P. aeruginosa* (Fig. 4.19 B), it can be observed that biofilm formation at 37^{o}C is higher in comparison to 25^{o}C, and *P. aeruginosa* shows the highest biofilm formation in comparison with *E. coli* at both temperatures. All Active Oxygen concentrations appear to be effective at reducing biofilm formation in comparison to the untreated samples, and effectiveness appears to be maintained across different dilutions. The most effective Active Oxygen concentration appears to be 10x concentrated. In addition, Product 1 appears to prevent biofilm formation in both temperatures, however the effect is less pronounced in comparison to *E. coli.*

4.2.12 Static planktonic growth removal assays of product combinations in nutrient rich media (LB) – Non S.O.P. experiments

This section contains the results obtained from the planktonic growth inhibition assays performed on two bacterial species, *Escherichia coli K12* and *Pseudomonas aeruginosa PA01* at two different temperatures, 25 and 37 degrees Celsius. The results represent the optical densities obtained after 24hrs of incubation in nutrient rich media with the addition of active oxygen at the normal working concentration + various eco3spa product dilutions and the absolute biofilm formation measured via crystal violet staining after a further 24hrs incubation. Growth medium was used as a blank.

Fresh media was added in the control wells and the products added were diluted in media.

It must be made clear that the researcher wished to show the data here, even though they were not optimized to show trends. In addition, due to experimental limitations, statistical analyses were not logical and were not performed.



Figures 4.20 A-D Planktonic growth of *E. coli K12* (Fig. 4.20 A-B) and *P. aeruginosa P*AO1 (Fig. 4.20 C-D) (indicated as OD595) in nutrient-rich media (LB) at 25°C (Fig. 4.20 A-C) and 37°C (Fig. 4.20 B-D) in the presence and absence of a range of product combinations with Product 1 (biofilm remover) and Product 2 (water conditioner). 'Normal' indicates usage of active

oxygen at SOP, other concentrations as indicated relate to normal SOP. Product 1 and Product 2 were used at SOP concentration only. N=3 for each data point. Error bars indicate standard error of the mean (SEM).

Untreated *E. coli* samples at 37^{o}C (Fig. 4.20 A-B) show the highest planktonic growth with an approximate endpoint reading of 1.4 AU, followed by *E. coli* at 25^{o}C with an approximate endpoint reading of 1.0 AU. In both temperatures, all product combinations appear to be effective in preventing planktonic growth to some degree, and the level of reduction appears to be similar across treatments. The best preventative effects for *E. coli* at 25^{o}C (Fig. 4.20 A) appears to be Product 1 by itself and Active Oxygen in SOP concentration by itself. At 37^{o}C (Fig.4.20 B), the best preventative effect appears to be Active Oxygen in SOP concentration in combination with Product 1, followed by Active Oxygen in combination with Product 2 (water conditioner).

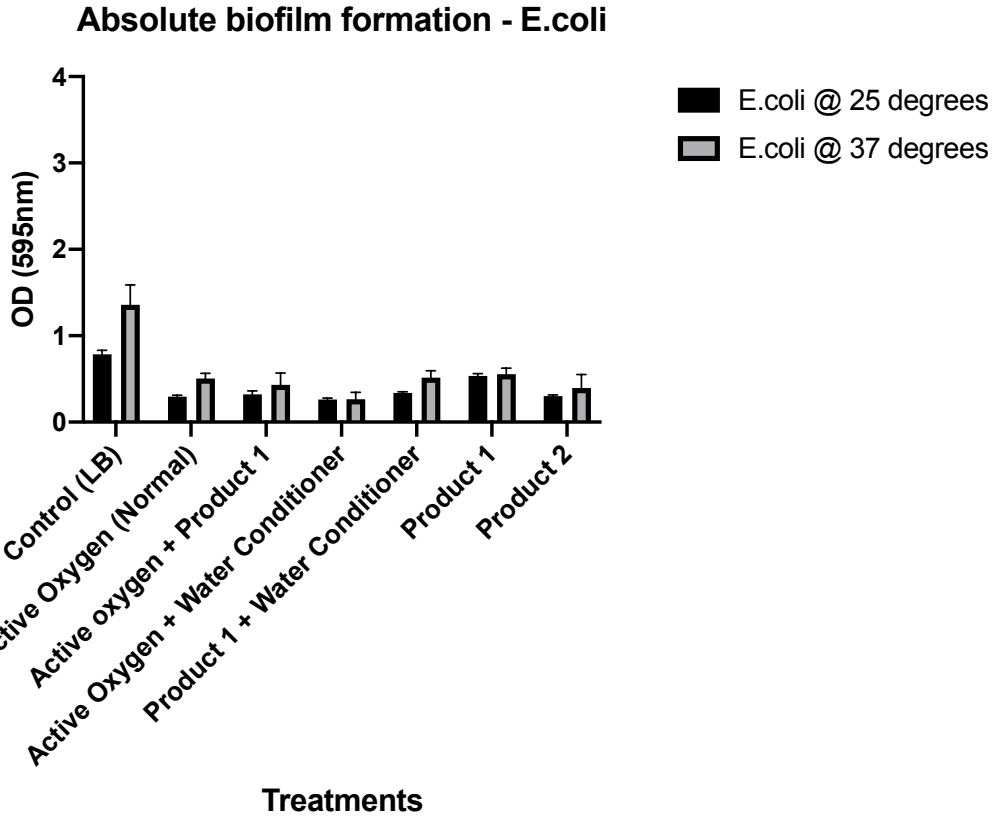
Untreated *P. aeruginosa* samples at 37^{o}C (Fig. 4.20 C-D) show the highest planktonic growth with an approximate endpoint reading of 1.1 AU, followed by *P. aeruginosa* at 25^{o}C with an approximate endpoint reading of 1.0 AU. In both temperatures, all product combinations appear to be effective in preventing planktonic growth to some degree, and the level of reduction appears to be similar across treatments, however all treatments appear to be more effective at 37^{o}C. The best preventative effects for *P. aeruginosa* at 25^{o}C (Fig. 4.20 C) appears to be Active Oxygen by itself. At 37^{o}C (Fig. 4.20 D), the best preventative effect appears to be Active Oxygen in combination with Product 1, followed by Active Oxygen by itself.

4.2.13 Static biofilm inhibition removal of product combinations in nutrient rich media – Non S.O.P. experiments

This section contains the results obtained from the static biofilm removal assays performed on two bacterial species, *Escherichia coli K12* and *Pseudomonas aeruginosa PA01* at two different temperatures, 25 and 37°C. The results represent the absolute biofilm formation (measured as OD at 595nm) obtained after 24hrs of incubation in LB nutrient rich media and a further 24hrs incubation (time of measurement) with the addition of various product combinations, followed by crystal violet staining.

Fresh media was added in the control wells and the products added were diluted in media.

It must be made clear that the researcher wished to show the data here, even though they were not optimized to show trends. In addition, due to experimental limitations, statistical analyses were not logical and were not performed.



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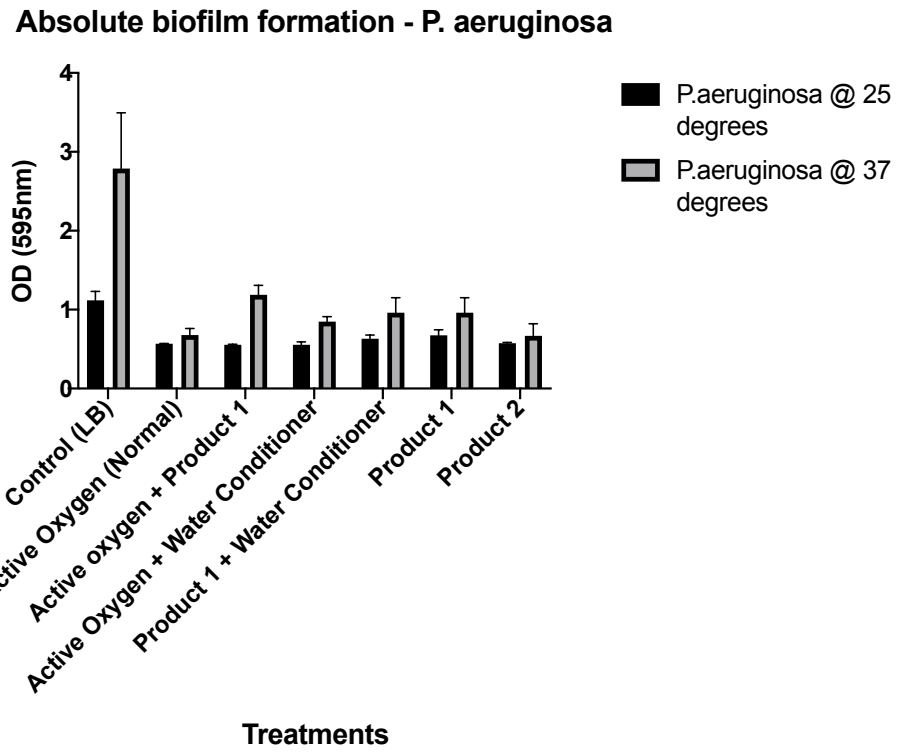


B

Figures 4.21 A and B Absolute biofilm formation (indicated as OD595) of *E. coli K12* (A) and *P. aeruginosa* PAO1 (B) in nutrient-rich media (LB) in the presence and absence of a range of product combinations with Product 1 (biofilm remover) and Product 2 (water conditioner), which were added after an initial 24hrs of growth. 'Normal' indicates usage of active oxygen at SOP, other concentrations as indicated relate to normal SOP. Product 1 was used at SOP concentration only. N=3 for each data point. Error bars indicate standard error of the mean (SEM).

By observing the absolute biofilm formation results for *E. coli* (Fig. 4.21 A) it can be seen that *E. coli* shows higher biofilm formation at 37°C compared to 25°C with average endpoint reading of around 1.4 AU in comparison to 0.8 AU. All product combinations appear to be effective to some degree at reducing biofilm formation in comparison to the untreated samples. The highest biofilm preventative effect for both temperatures is exhibited by Active Oxygen in combination with Product 2, followed by Product 2 by itself. In addition, generally less biofilm formation is observed at 25°C in comparison to 37°C.

By observing the absolute biofilm formation results for *P. aeruginosa* (Fig. 4.21 B) it can be seen that *E. coli* shows higher biofilm formation at 37°C compared to 25°C with average

endpoint reading of around 3.7 AU in comparison to 1.1 AU. All product combinations appear to be effective to some degree at reducing biofilm formation in comparison to the untreated samples. The highest biofilm preventative effect for both temperatures is exhibited by Active Oxygen by itself, followed by Product 2 by itself. Similar to the results for *E. coli* (Fig. 4.21 A), generally less biofilm formation is observed at 25°C in comparison to 37°C.

4.2.14-Colour change observations in *P. aeruginosa* overnight cultures

While removing the *P. aeruginosa* overnight cultures containing M9 minimal media pH adjusted to pH 7, pH 7.4 and pH 8 from the shaking incubator, before the preparation of dilutions for the titer plate assays, the color of the media was observed.

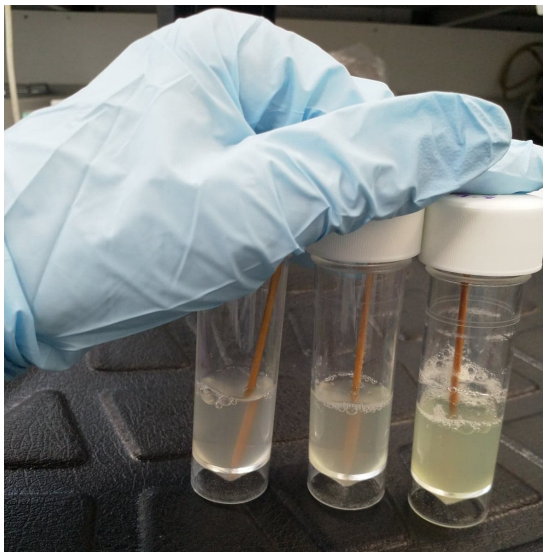


Figure 4.22: *P. aeruginosa* overnight cultures. From left to right: pH 7, pH 7.4 and pH 8

Of interest was the green color of the *P. aeruginosa* overnight (16 hrs.) shaken cultures at 37°C. In particular, the sample incubated at pH 8 appeared denser and with a more intense green color in comparison with the other pHs.

4.3 Discussion

4.3.1 Planktonic growth

Overall, in M9 media *E. coli* exhibited higher levels of planktonic growth in comparison with *P. aeruginosa* (Figs. 4.1 A-B, 4.2 A-B). *E. coli* reached higher levels of planktonic growth at 25°C when compared to 37°C, however this was not the case for *P. aeruginosa*, as higher temperatures (37°C) led to higher planktonic growth (Figs. 4.1 A-B, 4.2 A-B). Regarding the effect of pH, at pH 7, *E. coli* at both temperatures showed higher planktonic growth in comparison with *P. aeruginosa* at both temperatures (Fig, 4.7 A). At pH 7.4, *E. coli* and *P. aeruginosa* at 37°C surpassed bacteria at 25°C (Fig, 4.7 B). At pH 8, both strains showed similarly high levels of planktonic growth with the exception of *P. aeruginosa* at 25°C (Fig. 4.7 C). With regards to active oxygen, it appeared to be equally effective at preventing planktonic growth against both species in concentrations higher than 1 in 10 SOP (Figs, 4.1 A-B, 4.2 A-B). Moreover, it appeared equally effective in both temperatures tested, however a small decrease in activity in both species appeared at 25°C (Figs 4.1 B, 4.2 B). Regarding the effect of pH, active oxygen was effective across all pH tested, with a small decrease in activity at pH 8 and 25°C (Figs 4.1 B, 4.2 B). Furthermore, the addition of water conditioner by itself appeared to have a small yet significant effect in planktonic growth prevention in both strains and temperatures, and its combination with active oxygen appeared to neither increase not inhibit its activity for both strains, temperatures and all pH tested.

Regarding the experiments in LB, both strains exhibited high levels of planktonic growth (4.13 A-B, 4.14 A-B), with the highest planktonic growth observed by *E. coli.* Both strains exhibited the highest levels of growth at 37°C growth (4.13 A-B, 4.14 A-B). With regards to active oxygen, as it was not used in SOP conditions in the LB experiments, loss of activity in comparison to SOP conditions is noted. Regardless, active oxygen is still capable of bactericidal activity, with the most effective concentrations noted as the 1 in 10 and 10x concentrated preparations (Figs 4.13, 4.14, 4.15, 4.16). The water conditioner and biofilm

remover, although not bactericidal, appear to have an effect in preventing planktonic growth, especially at higher temperatures (37°C). The combination of water conditioner and active oxygen, once more, appeared to neither increase not inhibit its activity (Fig. 4.15, 4.16 and 4.20).

4.3.2 Planktonic growth – strain comparisons

In M9 media experiments, untreated *E. coli* exhibited higher levels of planktonic growth in comparison with untreated *P. aeruginosa* (Figs. 4.1 A-B, 4.2 A-B). In particular, *E. coli* planktonic growth at 37°C remained consistently high (Fig. 4.7 A-C). In contrast, P. *aeruginosa* planktonic growth remained in lower levels, however at 37°C and at higher pH the levels of planktonic growth began to approach those of *E. coli* (Fig 4.7 A-C). Moreover, in comparison with untreated wells, *E. coli* showed a more dramatic decrease in planktonic growth after treatment with the lowest active oxygen concentration (1 in 600 SOP) in comparison to *P. aeruginosa* (Fig. 4.7 A-C).

The high growth levels of *E. coli* growth in M9 media are not surprising, as it is a very commonly used *E. coli* growth medium (Thakur et al., 2010). In addition, *E. coli* cells are well-known for their capability of growing in glucose minimal media, and it has been demonstrated that growth in minimal media can even provide *E. coli* cells with survival benefits, such as increased transcription of genes which can support survival, such as stress proteins (Tao et al., 1999). In addition, *E. coli K12* is known for its susceptibility to disinfectants (Berney et al., 2006)

4.3.3 Planktonic growth – temperature comparisons

E. coli reached higher levels of planktonic growth in M9 minimal media at 25°C when compared to 37°C, however this was not the case for *P. aeruginosa*, as higher temperatures (37°C) led to higher planktonic growth (Figs. 4.1 A-B, 4.2 A-B). This was surprising, as it was expected that higher planktonic growth would be observed at 37°C, which is the optimum temperature for *E. coli* growth (Doyle and Schoeni, 1984). However, there are factors which may enable and even promote growth in lower temperatures, such as cold shock protein D (Langklotz and Narberhaus, 2011).

CspD (cold shock protein D) may play a role in inhibiting planktonic growth in minimal media. CspD is a toxin protein expressed in upon entry into stationary phase and upon carbon starvation (Langklotz and Narberhaus, 2011). The family of cold-shock proteins are commonly RNA chaperones involved in a variety of functions, supercoiling of DNA to initiation of translation, and combat the formation of stable secondary structures in mRNAs to aid translation at low temperatures (Jones and Inouye, 1994). However, unlike most cold-shock proteins, CspD acts as a replication inhibitor due to ssDNA binding (Yamanaka and Inouye, 1997). It has been shown that CspD expression limits growth of *E. coli K12* in M9 minimal media at 30^{o}C (Langklotz and Narberhaus, 2011), which may partially explain the results of this project (low levels of M9 and LB growth at 37°C) and in addition, CspD has been shown to be poorly degraded when cells grow in low temperatures (30^{o}C) in both LB and M9 media, in contrast with the optimum temperature of 37^{o}C (Langklotz and Narberhaus, 2011).

A study has shown that CspD is regulated by Cyclic AMP (cAMP) receptor protein (CRP), a regulator involved in the induction of genes during the stationary phase in *E. coli* (Uppal et al., 2014). cAMP is mainly involved in catabolite repression in bacteria and plays a large role in *E. coli* metabolism regulation; the uptake of *E. coli* preferential carbon sources, such as glucose, requires the phosphoenolpyruvate: sugar phosphotransferase system (PTS) (Deutscher et al., 2006). When PTS sugars are available, the phosphoryl group of phosphorylated EIIA protein (EIIA∼P) is transferred to PTS sugars to perform the uptake of those sugars into the cell (Park et al., 2006). When PTS sugars are not available and non-PTS sugars are available in the medium, adenylyl cyclase CyaA is activated by EIIA∼P leading to cAMP synthesis (Park et al., 2006). Following that, cAMP-CRP activates the transcription of genes encoding proteins responsible for the transport of non-PTS sugars, resulting in the cell up-taking non-PTS sugars (Park et al., 2006).

4.3.4 Planktonic growth – media and time comparisons

M9 minimal media is a widely used buffered growth medium which supports *E. coli* planktonic growth and contains inorganic salts and a carbon source; in this case, glucose (Naves et al., 2008). Luria-Bertani media is widely used for *E. coli* growth and research, and its rich nutrient and oligopeptide content provides an ideal growth environment (Sezonov, Joseleau-Petit and D'Ari, 2007). Interestingly, after 48hrs incubation in both temperatures, *E. coli* exhibited

higher planktonic growth in M9 minimal media in comparison to LB (Fig. 4.14 A-B and 4.18 A). Although *P. aeruginosa* showed low levels of planktonic growth in M9 after 24 hrs. (4.9 A-B), after 48 hrs. the planktonic growth for both temperatures reached a similar value (4.18 C-D). Similar to *E. coli*, *P. aeruginosa* showed higher planktonic growth in M9 minimal media in both temperatures in comparison to LB after 48hrs incubation (Fig. 4.14 A-B and 4.21 B)

This is not surprising, as *P. aeruginosa* reaches significant levels of planktonic growth in both LB and M9 minimal media at 37^{o}C (LaBauve and Wargo, 2012). In addition, studies have showed that *P. aeruginosa* shows increased planktonic growth in LB medium in comparison to M9 at 30^{o}C, which may partially explain the difference in planktonic growth at 25^{o}C, as many metabolic pathways shut down at temperatures below 30^{o}C (LaBauve and Wargo, 2012 (Schleheck et al., 2009).

In addition, similar to *E. coli*, *P. aeruginosa* also exhibits RpoS-mediated stress tolerance (Jorgensen et al., 1999). RpoS has been shown to provide *P. aeruginosa* with tolerance to prolonged starvation in glucose-supplied media, such as the M9 media used in this project, which may explain the increase in growth (Jorgensen et al., 1999).

Of interest is also the metabolism of *E. coli* growing in LB media. A study has shown that over a growth period of about 8 hours, *E. coli* switches from a sequential mode of substrate utilization to the simultaneous one. The first carbon substrates utilized are maltose and maltodextrins, followed mannose, galactose, fucose, rhamnose, mannitol, trehalose, and arabinose (Baev et al., 2006).

In order to explain the results obtained after long periods of incubation, during which the cells were in the stationary phase, the understanding of the role of sigma factors is crucial. In order for bacterial cells to regulate the expression of genes necessary for routine cell functions or adaptation to stress conditions, many regulatory mechanisms are needed. The most important mechanism in gene regulation involves the initiation of transcription, where the DNA-dependent RNA polymerase (RNAP) is the main player (Wosten, 1998). RNAP is the catalytic protagonist in regards to the RNA synthesis from a DNA template, however it is incapable of initiating transcription by itself and an additional polypeptide known as a σ-factor is required (Polyakov et al., 1995; Travers and Burgessrr, 1969). Sigma-factors belong to a

family of small proteins that can bind reversibly with the RNAP core enzyme. Together, the σ-factor and the RNAP core enzyme form an initiation-specific enzyme, the RNAP holoenzyme (Travers and Burgessrr, 1969).

Of particular importance to this project is the stationary phase σ-factors, such as RpoE and RpoS, found in both *E. coli* and *P. aeruginosa,* and is responsible for mediating the general stress response (Wosten, 1998). RpoE is only found in *E. coli,* while RpoS RpoS is unstable during exponential phase but stable during the stationary phase, and is upregulated by both positive regulators such as ppGpp and polyphosphate and negative regulators such as cAMP (Jaishankar and Srivastava, 2017). RpoS is responsible for inducing a series of genes which cause starved *E. coli* cells to become smaller, their cell walls to become more highly cross-linked, condense their cytoplasm and increase of their periplasmic volume, which studies have shown that these effects take place in *E. coli* in rich medium at 37^{o}C at an OD of 1 (Lange and Hengge-Aronis, 1991; Zambrano and Kolter, 1996). RpoS leads to the development of a highly resistant state and allows the cells to not only overcome the initial stress encountered, but also to become tolerant to further stress conditions (Battesti et al., 2011) Studies have shown that RpoS is not only induced by entry into stationary phase, but by also high cell density (Liu et al., 2000). In addition, a recent study has shown that RpoS may be linked in the dynamic of persister cell formation and that ppGpp, a cyclic nucleotide acting as a secondary messenger in a similar fashion to c-di-GMP and cAMP which is upregulated by stress, plays an important role in RpoS activity (Kalia et al., 2013; Patange et al., 2018).

Prolonged starvation in the stationary phase, which could be the case for results obtained after 48hrs incubation, has been shown to result in Growth Advantage in Stationary Phase (GASP) phenotype. An interesting results from a study showed that older cell populations in stationary phase exhibiting GASP phenotype had a growth advantage, as they could utilize the nutrients released by dying cells (Zambrano and Kolter, 1996). This allows the cells to keep growing under starvation, and eventually allow them to replace the parental population (Jaishankar and Srivastava, 2017).

Interestingly, RpoS has also been found to play a role during exponential growth in *E. coli K12* in rich media (LB). Although growth in nutrient rich media is not likely to be stressful, the study showed that some stress-protective factors (osmY and gadAB) were maintained at low

levels during exponential growth, possibly allowing enhanced adaptation to changing environments and may play a protective role against osmotic and acid stress during this critical phase, in addition to genes related to carbon source transport, protein folding and iron acquisition (Dong et al., 2008).

4.3.5 Planktonic growth – pH comparisons

Regarding the effect of pH, at pH 7, *E. coli* at both temperatures showed higher planktonic growth in comparison with *P. aeruginosa* at both temperatures (Fig, 4.7 A). At pH 7.4, *E. coli* and *P. aeruginosa* at 37°C surpassed bacteria at 25°C (Fig, 4.7 B). At pH 8, both strains showed similarly high levels of planktonic growth with the exception of *P. aeruginosa* at 25°C (Fig. 4.7 C). *E. coli* at both temperatures showed the highest planktonic growth at pH 7 (Fig. 4.1 A and B). In addition to pH 7, *E. coli* at both temperatures also showed high levels of planktonic growth at pH 8, with endpoint measurements quite close to those for pH 7 (Fig. 4.1 A and B). *P. aeruginosa* showed the highest planktonic growth at pH 8 and the lowest at pH 7 (Fig. 4.2 A-B).

The results regarding *E. coli* planktonic growth make sense, as a study has shown that *E. coli* K12 doubling time at 37^{o}C is at its fastest at pH 7 at 18 minutes, in contrast to 25 minutes at pH 5 and 8.7 (Maurer et al., 2005). This also makes sense as the pH in the human gut, where *E.coli* is normally found, ranges from pH 6 to pH 7.4 (Fallingborg, 1999). In addition, the growth and adaptation of *E. coli* in mildly acidic conditions have been well-studied; growth in mild acid (pH 6-7) can enhance elements of cellular metabolism and cause upregulation of protein pumps and outer membrane proteins, which in turn increase influx of nutrients and thus aid growth (Harden et al., 2015). In addition, mildly acidic conditions allow the uptake of permeable acids, which acidify the cytoplasm, increase anions, and lower the cytoplasmic pH. This has been shown to induce a number of genes, such as genes associated to flagellar motility and oxidative stress (Maurer et al., 2005). It is known that *E. coli* in the human body can withstand pH from 2 to 8, and even harsh conditions caused by pancreatic secretions, including a pH of 10 (Maurer et al., 2005). Therefore, it would make sense for *E. coli* to exhibit resistance and capability to grow under higher pH values (Fig. 4.1 A and B). It is also known that *E. coli* growth using glucose causes acetate to accumulate, which is inhibitory for growth. An interesting study using LB media supplemented with glucose at different pH found that at

pH 7 and 8 a drop in media pH was noted, but surprisingly the metabolism of the cells re-calibrated it to values similar to the initial ones, which may explain the growth levels observed (Sánchez-Clemente et al., 2018). Rather surprisingly, untreated *E. coli* showed relatively low levels of planktonic growth at pH 7.4, especially at 25^{o}C.

A study has shown that *P. aeruginosa PA01* growth at low pH values, even at around pH 6 are growth inhibitory, which agrees with planktonic growth results for *P. aeruginosa* at pH 7 (Fig.4.7 A) (Bushell et al., 2018). Reasons why low pH may inhibit growth include lowering of cytoplasmic pH, anion toxicity and osmotic stress (Bushell et al., 2018). The highest planktonic growth in both temperatures was noted at pH 8, followed by pH 7.4 (Fig. 4.2 A and B). This comes in agreement with previous research, where *P. aeruginosa* exhibited slow growth in pH values below 6, but grew at an increased rate at pH 7.5, with a peak at around pH 8.2 (Tsuji et al., 1982).

Interestingly, at pH 8, *P. aeruginosa* shaken cultures exhibited a bright green color (Fig. 4.25. Phenazines are well-known for their bright blue-green pigment and have been shown to change color with pH change, which makes them useful in biosensors (Pierson and Pierson, 2010). In addition, a pigment produced by *P. aeruginosa PAO1,* pyoverdine, is responsible for the generation of a bright green pigment. It has been shown that *P. aeruginosa* at high pH values (around pH 8) shows enhanced pyoverdine production, which may explain the color change at high pH in this project (Albesa et al., 1985). RpoS is also responsible for pyocyanin production, and experiments have shown that *P. aeruginosa PA01* RpoS mutants were overproducing pyocyanin, while the overproduction of RpoS resulted in a decrease in pyocyanin levels (Suh et al., 1999).

4.3.6 Planktonic growth – effect of different products

In general, across all media used, the Active Oxygen product is effective at reducing bacterial growth to some or large degree, even when used at non-SOP recommendations. Both *E. coli* and *P aeruginosa* exhibited similar levels of susceptibility to Active Oxygen, with no planktonic growth observed at all at 37^{o}C in SOP experiments, in concentrations higher than 1 in 10 of SOP in M9 minimal media (Fig. 4.1 A and 4.2 A). However, some loss of activity was noted for both organisms at 25^{o}C, and in particular at pH 8 (4.1 B and 4.2 B).

This comes partially in agreement with a previous study on the efficacy of Virkon on both organisms, where it was discovered that the MIC for both *E. coli* and *P. aeruginosa* was the same (Gasparini et al., 1995). As Active Oxygen is well-known for its reactivity towards organic compounds (Bailey, 1958), it is possible that it may react with the organic nutrients present in LB media, such as yeast extract (Sezonov, Joseleau-Petit and D'Ari, 2007). It is possible that these reactions may lead to some loss of efficacy, which may explain the higher levels of activity noted in M9 media, where less organic nutrients are present (Rugbjerg, Feist and Sommer, 2018). This may also help explain the high levels of planktonic growth prevention achieved during the SOP experiments in M9 media (Section 4.2.1). Regarding the SOP experiments, the European Chemicals Agency mentions that the degradation of pentapotassium peroxymonosulfate is dependent on pH and temperature, with increased degradation noted at increasing temperatures and pH (ECHA, 2021). Most importantly, it was noted the substance has a half-life of above 800 h (at 20°C) in a buffered solution of pH 4, the half-life at pH 7 is 145 hours and only 2.8 hours at pH 9 (ECHA, 2021). Therefore, the loss of activity noted at low temperature (25^{o}C) and high pH makes sense. It also may be possible that the temperature of 37^{o}C has a beneficial effect on the activity and maintenance of Active Oxygen, but no literature was found on this particular topic. In conclusion, active oxygen appeared to be an effective planktonic growth inhibitor in SOP concentrations higher than 1 in 10 SOP against both strains under all conditions tested.

4.3.7 Planktonic growth – effect of product combinations

With regards to the combination of Active Oxygen with the water conditioner (Product 2), statistical analysis showed that it makes no difference in planktonic growth prevention (Fig. 4.9 A-B and 4.10 A-B). Scheirer-Ray-Hare analysis showed that Active Oxygen by itself was responsible for the prevention of planktonic growth, and no interactions with Product 2 were noted. A small, yet statistically insignificant increase in planktonic growth at the 1 in 10 Active Oxygen SOP concentration in combination with Product 2 was noted across all strains and temperatures (Fig. 4.9 A-B, 4.10 A) with the exception of *P. aeruginosa* at 25^{o}C (Fig. 4.10 B). It is not certain if some components of the Conditioner are responsible for this increase in endpoint measurements or whether a morphological change in surviving bacteria is responsible. According to the manufacturer, the water conditioner contains coconut fatty

acids and glycosides, and it may be possible that the surviving bacteria may be using some of the components as an additional or preferential nutrient source.

4.3.8 Biofilm formation

Overall, in M9 media and SOP experiments *P. aeruginosa* exhibited dramatically higher levels of biofilm growth in comparison with *E. coli* (Figs.4.8 A-C). *P. aeruginosa* reached higher levels of biofilm formation at 37°C when compared to 25°C, and higher temperature (37°C) led to *E. coli* increased biofilm formation (Figs.4.8 A-C). Regarding the effect of pH, at pH 7, *P. aeruginosa* at both temperatures showed higher planktonic growth in comparison with *E. coli* at both temperatures (Fig, 4.8 A). At pH 7.4, P. *aeruginosa* at both temperatures surpassed *E. coli* (Fig, 4.8 B). At pH 8, once more *P. aeruginosa* showed dramatically high levels of biofilm formation in comparison with *E. coli* (Fig. 4.8 C). With regards to active oxygen, it appeared to be equally effective at preventing biofilm formation against both species in concentrations higher than 1 in 10 SOP (Figs, 4.3 A-B, 4.3 A-B). Moreover, it appeared equally effective in both temperatures tested, however a small decrease in activity in *E coli* appeared at 37°C (Fig 4.3 A). Regarding the effect of pH, active oxygen was effective across all pH tested, with no decrease in activity noted at any pH or temperature at concentrations higher than 1 in 10 SOP (Figs 4.8 A-C). Furthermore, the addition of water conditioner by itself appeared to have a small yet significant effect in planktonic growth prevention in both strains and temperatures (Fig. 4.11 A-B, 4.12 A-B), and its combination with active oxygen appeared to neither increase not inhibit its activity for both strains, temperatures and all pH tested.

Regarding the experiments in LB, both strains exhibited high levels of biofilm formation after 48 hrs. (4.17 A-B), with the highest planktonic growth observed by *P. aeruginosa.* Both strains exhibited the highest levels of biofilm formation at 37°C (4.17 A-B). With regards to active oxygen, as it was not used in SOP conditions in the LB experiments, loss of activity in comparison to SOP conditions is noted. Regardless, active oxygen is still capable of inhibiting biofilm formation to some degree, with the most effective concentrations noted as the 10x concentrated preparation (Figs 4.17 A-B). The water conditioner and biofilm remover, although not bactericidal, appear to have an effect in preventing planktonic growth by

themselves, especially at higher temperatures (37°C) (Fig. 4.21). The combination of water conditioner and active oxygen, once more, appeared to neither increase not inhibit its activity (Fig. 4.21).

4.3.9 Biofilm formation – strain comparisons

In M9 media experiments, untreated *P. aeruginosa* exhibited higher levels of biofilm formation in comparison with untreated *E. coli* (Figs. 4.8 A-C). In particular, *P. aeruginosa* biofilm formation at 37°C remained consistently the highest overall (Fig. 4.8 A-C). In contrast, *E. coli biofilm* formation remained in lower levels (Fig 4.8 A-C). Moreover, in comparison with untreated wells, *P. aeruginosa* showed a more dramatic decrease in planktonic growth after treatment with the lowest active oxygen concentration (1 in 600 SOP) in comparison to *E. coli* (Fig. 4.8 A-C).

The high growth levels of *P. aeruginosa* growth in M9 media are particularly interesting. First of all*, P. aeruginosa* PA01 is known to form biofilm equally well in both LB and M9 media (Schleheck et al., 2009). However, it could be that the limited nutrients may induce the transcription of stress factors such as RpoS, can provide *P. aeruginosa* biofilms with survival benefits, such as resistance to disinfectants and higher density (Bouillet et al., 2019). In addition, *P. aeruginosa* is known for its biofilm production and for its property to grow biofilm throughout the well tested (Ochoa et al., 2015; (Merritt et al., 2005).

4.3.10 Biofilm formation – temperature comparisons

Regarding the effect of temperature on biofilm formation, in M9 minimal media and SOP experiments, *P. aeruginosa* showed dramatically increased biofilm formation at 37°C in comparison to 25°C (Fig 4.8). The same can be observed with *E. coli,* with higher biofilm formation in the same experiment noted at 37°C (Fig. 4.8). With regards to growth in LB media after 48hrs, the highest biofilm formation was noted by *P. aeruginosa* at 37°C (around 6.000 AU) (Fig, 4.17). *E. coli* also exhibited the highest biofilm formation at the same temperature (Fig 4.17). However, in all media tested, the difference temperature makes is more dramatic in *P. aeruginosa* in comparison with *E. coli,* where the difference in biofilm formation between the two temperatures is smaller.

The large amounts of biofilm noted at 37°C are not surprising, as studies have shown that not only *P.aeruginosa* shows increased biofilm formation at this temperature, but also that biofilm biomass, EPS production, adhesion and mechanical stability were highest at 37°C in comparison with lower temperatures such as 28°C (Donnarumma et al., 2010; Kannan and Gautam, 2015).

Regarding the biofilm formation of *E. coli* at lower temperatures, CspD has been linked to biofilm formation in *E. coli* as well as the formation of persister cells (Kim et al., 2010). The term persisters is used to describe bacterial cells under stress, such as starvation or use of antimicrobials, and under this resting state they exhibit resistance to nearly all antibiotics without undergoing genetic change (Wood, 2017). Importantly, most of these cells are found in biofilms in humans, which highlights the importance of understanding and fighting against them (Wood, 2017). It has been demonstrated that CspD is regulated by Cyclic AMP (cAMP) receptor protein (CRP), a regulator involved in the induction of genes during the stationary phase in *E. coli* (Uppal et al., 2014)Additionally, cAMP-CRP is involved in the transcription of about 7% of *E. coli* genes, including genes linked to biofilm formation.

4.3.11 Biofilm formation – media and time comparisons

M9 minimal media is a widely used buffered growth medium which supports *E. coli* planktonic growth and contains inorganic salts and a carbon source; in this case, glucose (Naves et al., 2008). Luria-Bertani is widely used for *E. coli* growth and research, and its rich nutrient and oligopeptide content provides an ideal growth environment (Sezonov, Joseleau-Petit and D'Ari, 2007). Therefore, it would be expected that *E. coli* would exhibit higher absolute biofilm formation after 24hrs in LB media in comparison to minimal media, which was the case for both temperatures (4.19 A and 4.9 A-B). Interestingly, after 48 hrs. incubation, *E. coli* exhibited absolute biofilm formation endpoint values in LB media quite close to those observed after 48hrs incubation in minimal media (Fig. 4.15 A and 4.17 A). *P. aeruginosa* shows higher biofilm formation in M9 media after 24hrs at 37^{o}C (Fig. 4.10 A and 4.19 B), but not at 25^{o}C (Fig. 4.10 B and 4.19 B). Interestingly, after 48hrs incubation, *P. aeruginosa* at 25^{o}C exhibited absolute biofilm formation endpoint values in LB media quite close to those

observed after 48hrs incubation in minimal media, but not at 37^{o}C where higher endpoint values were noted at LB media (Fig. 4.15 B and 4.17 B).

Increased levels of *E.* coli biofilm in nutrient rich media has been observed in previous studies (Pratt and Kolter, 1998).In addition, previous studies have confirmed that E. coli biofilm adherence/formation increases under nutrient low conditions (Reisner et al., 2006). Furthermore, growth in M9 media has been shown to provide some benefits to biofilms, such as resistance to physical forces (Jefferson, 2004). Additionally, the growth rate of cells plays a major role in biofilm survival. Studies have shown that the sensitivity of biofilm cells to antibiotics increased in tandem with growth rate, suggesting that a slower growth rate may play a protective role against antimicrobials (Evans et al., 1991). Moreover, the role of RpoS in biofilm formation cannot be ignored. As mentioned in the previous section, RpoS is an important mediator during stress response, evidenced by the fact that RpoS mutants are incapable of forming mature biofilms, hinting that RpoS is needed for the expression of genes expressed in biofilms (Collet et al., 2008). Indeed, RpoS mutants showed impaired surface growth and biofilm architecture (Collet et al., 2008), which further highlight the role of stress mediators in biofilm formation.

P. aeruginosa strains are known for being resilient biofilm formers under varying conditions (Ochoa et al., 2015). In addition, *P. aeruginosa* PA01 has been shown to form biofilm equally well in both LB and M9 media, which may explain the similar values notes in this project (Schleheck et al., 2009).

4.3.12 Biofilm formation – pH comparisons

pH plays a large role in biofilm formation and this is reflected in the biofilm formation results for various pH values obtained during this project. Regarding untreated samples, *E. coli* showed the highest absolute biofilm values at pH 7.4 for both 25^{o}C and 37^{o}C (Fig. 4.3 A and B). This comes in agreement with the results of a study which came to similar conclusions, with *E.coli* showing high biofilm formation at pH 7.4 at 25^{o}C (Mathlouthi et al., 2018). Interestingly, *E. coli* maintains its internal pH at around 7.6, which may also help explain the increased biofilm formation (Maurer et al., 2005). At high pH values, *E. coli* showed very low biofilm formation (Fig. 4.8 C). Studies have shown that high pH (8.7) represses flagellar and

chemotaxis genes and represses synthesis of flagella, which may have an effect on biofilm (Maurer et al., 2005).

pH is also of importance in *P. aeruginosa* biofilm formation. Across all temperatures and treatments, *P. aeruginosa* exhibited the highest biofilm formation at pH 8, followed by pH 7.4, which comes in agreement with the results of a study assessing biofilm formation at different pH values, where accelerated biofilm formation was observed at pH 8 and 7.4 in comparison to pH 5.5, using crystal violet quantification (Hostacka et al., 2010). In addition, pH has an effect on the expression of *P. aeruginosa* virulence factors, such as alginate. Indeed, a study has found increased alginate production in *P. aeruginosa* at pH 8, which consequently leads to increased biofilm formation at higher pH (Harjai et al., 2005). The lowest biofilm formation was noted at pH 7. Low biofilm formation by *P. aeruginosa* at low pH has been observed before, and in a study by Stoodley et al, *P. aeruginosa* biofilm thickness fell to 68% at pH 3 (Stoodley et al., 1997).

RpoS also plays an important role in regulating *P. aeruginosa* biofilms. A study has shown that RpoS has a positive effect on biofilm formation, as RpoS mutants showed a significant decrease in biofilm thickness and presence of more planktonic cells (Bouillet et al., 2019). In addition, RpoS is important in *P. aeruginosa* antibiotic resistance, as mutants were more susceptible to antibiotics in comparison with wild-type (Whiteley et al., 2001). A study has shown that RpoS also regulates quorum sensing, and RpoS mutants showed increased levels of rhlI, which is associated with Pel synthesis (Whiteley et al., 2000). Therefore, it could be linked to the fact that *P. aeruginosa* at pH 7 and 7.4 showed lower biofilm formation (Fig. 4.4 A and B).

4.3.13 Biofilm formation – effect of different products

Both *E. coli* and *P aeruginosa* exhibited similar levels of susceptibility to Active Oxygen, with no or very little absolute biofilm formation observed at all at 37^{o}C in SOP experiments, in concentrations higher than 1 in 10 of SOP in M9 minimal media (Fig. 4.3 A-B and 4.4 A-B). Interestingly, pH and temperature did not appear to play a major role in influencing the activity of Active Oxygen, with the exception of *E. coli* at both temperatures, where very small amounts of biofilm were noted (Fig 4.3 A-B). Rather surprisingly, almost no *P. aeruginosa*

biofilm at all was noted in both temperatures in all concentrations higher than 1 in 10 SOP (Fig. 4.4 A-B). Product 2 (water conditioner) used by itself showed to have a small yet significant effect at preventing biofilm formation in both organisms and temperatures (Fig. 4.11 A-B, 4.12 A-B). Interestingly, although not marketed as a biofilm preventing agent, Product 1 (biofilm remover) also had an effect in biofilm prevention, which was more prominent in *E. coli* (Fig. 4.19 A-B).

It is possible that the small amounts planktonic growth noted for both organisms at 25^{o}C, and in particular at pH 8 (4.1 B and 4.2 B), may be at the viable-but-not-culturable state and may be incapable of producing biofilm, as no biofilm was measured for the same treatments (Li et al., 2014). Regarding the biofilm remover, according to the sponsoring company it contains a blend of surfactants. This would justify the reduction in biofilm formation, especially in *E. coli*, as surfactants have been shown to inhibit biofilm formation at the air-liquid and solid-liquid interface (Wu et al., 2013). In addition, surfactants have been demonstrated to prevent planktonic cells from attaching to the preformed biofilm in both *E. coli* and *P. aeruginosa* (Davey et al., 2003).

4.3.14 Biofilm formation – effect of product combinations

According to the sponsoring company, the water conditioner contains a blend of enzymes, coconut extract, pH stabilizers and minerals. The combination of active oxygen and water conditioner (Product 2) did not appear to either enhance or inhibit the biofilm preventing properties of the tablets (Fig. 4.11 A-B, 4.12 A-B). Overall, the addition of Product 2 does not appear to inhibit or further enhance the biofilm prevention ability of Active Oxygen, which was confirmed by a series of Scheirer-Ray-Hare tests. Interestingly, however, at 25^{o}C in both *E. coli* and *P. aeruginosa* there was some increased biofilm formation noted in the combination of Active Oxygen in the 1 in 10 SOP concentration plus Product 2, compared to Active Oxygen in the same concentration by itself.

To date, information on the synergistic effect of an enzymatic product and Active Oxygen was not found. It may be possible that in sub-inhibitory stress conditions the addition of Product 2 at 25^{o}C could lead to survival of species and possible development of resistance, which may have an effect on virulence and pathogenicity. A possible theory which

marposexplain the phenomenon may include the need for high temperature for sufficient "activation" of the water conditioner as the product is added when the hot tub is operational, and the water has reached 37^{o}C. It is possible that low temperature and stress conditions generated by the sub-inhibitory concentration of Active Oxygen may cause the bacteria to utilize some of the components of the water conditioner as environmental nutrients, which may explain the increase in biofilm formation.

The water conditioner may also assist the biocidal effect of Active Oxygen by acting as a pH regulator. In a brief experiment, the addition of Active Oxygen in distilled water reduced its pH from approximately 5.5 to 3 (data not shown). Indeed, Virkon preparations have been shown to have a low pH of around 2.6 – 3, which agrees with this observation (Hernndez et al., 2000) As the company's description of the conditioner mentions is acts as a pH regulator, it can be hypothesized that Active Oxygen may act more efficiently at certain pH ranges.

Chapter 5: Initial studies of effect of Active Oxygen and Conditioner on cell morphology and viability

5.1 Introduction

In Chapter 4, SOP experiments in minimal media showed that Active Oxygen, when used in concentrations higher than 1 in 10 SOP, was effective at preventing planktonic growth and biofilm formation across all pH tested and at both 25°C and 37°C. Moreover, the addition of water conditioner appeared to neither significantly enhance nor inhibit the activity of the sanitizing tablets. This led to the consideration that the water conditioner may affect cell morphology and viability, leading to the decision for undertaking light microscopy and live-dead staining experiments. In addition, Log kill experiments are presented in this chapter, which were of great interest to the sponsoring company. It is important to note that work in this chapter was undertaken in the last 2 weeks of the researcher's lab time, under time constraints, lab equipment limitations and under n=1 conditions.

5.2 Cell morphology

In the previous chapter, experiments showed that the addition of water conditioner appeared to neither significantly enhance nor inhibit the activity of the sanitizing tablets. Experiments involving combinations of active oxygen with the conditioner in sub-optimal concentrations (1 in 10 SOP concentration) led to a small yet no significant increase in OD measurements and biofilm formation. This led to the question of whether this spike in OD is caused by cell clumping or changes in cell morphology. In addition, the paper of Stevenson et al. showed that cells under stress undergo morphological changes which may further influence OD measurements (Stevenson et al., 2016). 10 µl of sample were pipetted straight from a microtiter plate well on a microscope slide without dilution. Figure 5.1 shows micrographs of the effect(s) of Active Oxygen by itself or in combination with the Water Conditioner on the cell morphology of *E. coli* K12 grown in M9 at 37^{o}C at pH 7.4 for 24hrs. Figures 4.1 A and 4.2 A show the results of planktonic growth and biofilm formation corresponding to each treatment.

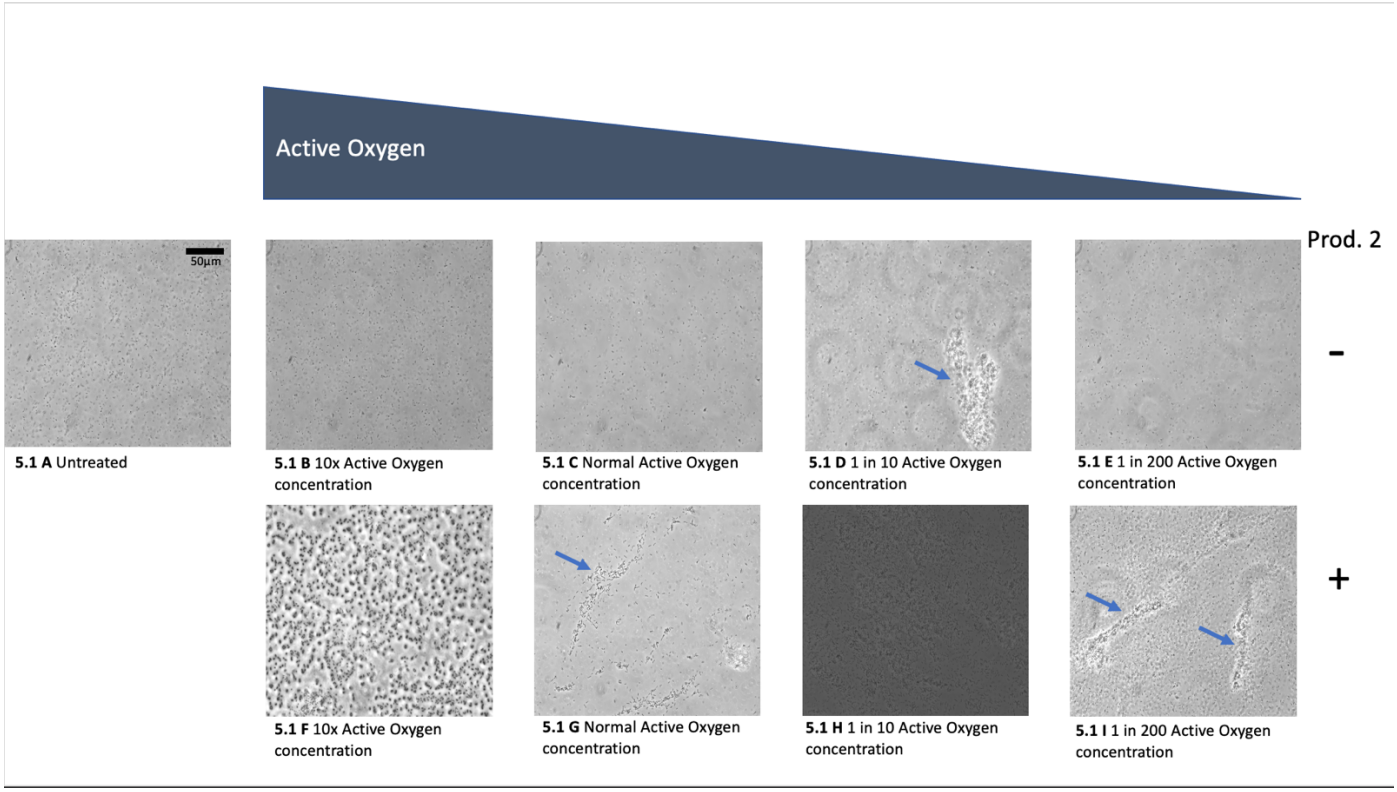


Figure 5.1. Effect of increasing concentrations of active oxygen on the cell morphology of *E. coli* K12 grown for 24h in M9 pH 7.4 at 37°C in the absence (upper panels) or presence (lower panels) of water conditioner (Product 2). Arrows point to visible cell clumps. Bar indicates 50µm.

Figure 5.1 shows that relatively high cell densities were reached in each well. This was partially because the samples used were undiluted, even though the actual measurements of cell densities (OD595) indicated reductions in the presence of active oxygen (Chapter 4, Figs 4.1 A and 4.9 A). In addition, motile cells were observed in the control and across all experiments containing only Active Oxygen. Interestingly, the addition of water conditioner (Product 2) in combination with Active Oxygen resulted in a decrease in cell motility, with a concomitant increase in large numbers of immotile cells (data not shown as no micrograph taken). The combination of Active Oxygen at the Normal concentration with Product 2 resulted in *E. coli* forming chain-like clumps. Treatment of cells with one-tenth strength Active Oxygen resulted in the formation of cell clumps, more of which are visible after the addition of Product 2. The combination of Active Oxygen at the lowest tested concentration with Product 2 also resulted in the formation of long, clump-like structures. It appears that the addition of Product 2 led to enhanced “clumping”, though it remains to be established if one or multiple components

in product 2 was responsible for this phenotype. Another question generated from these observations was whether the cells in the “clumps” were alive, viable-but-not-culturable, or dead, which could potentially affect efficacy of water sanitation at the longer term.

5.3 Fluorescence microscopy images

To gain an appreciation of the viable state of cells in clumps in the presence of Product 2 it was decided to perform Live-Dead staining/fluorescent microscopy. Propidium iodide (dead stain) and SYTO 9 (live stain) were pipetted into a well containing the samples and the well contents were thoroughly mixed. For the Negative control, a preparation of 5ppm chlorine was used. 9µl of sample were pipetted straight from a microtiter plate on a microscope slide with no dilution.

As the experiment took place in the last 2 weeks of research with limited support and equipment limitations, this caused the late and n=1 nature of these experiments, lack of computer-controlled image capture (leading to suboptimal image capture via mobile phone camera). Additionally, lack of experience with this type of instrumentation, led to movement of slides for images that otherwise should not be moved to be fully comparable, allowing overlays. This also explains the lack of light-field micrographs to indicate all cells in the field of vision.

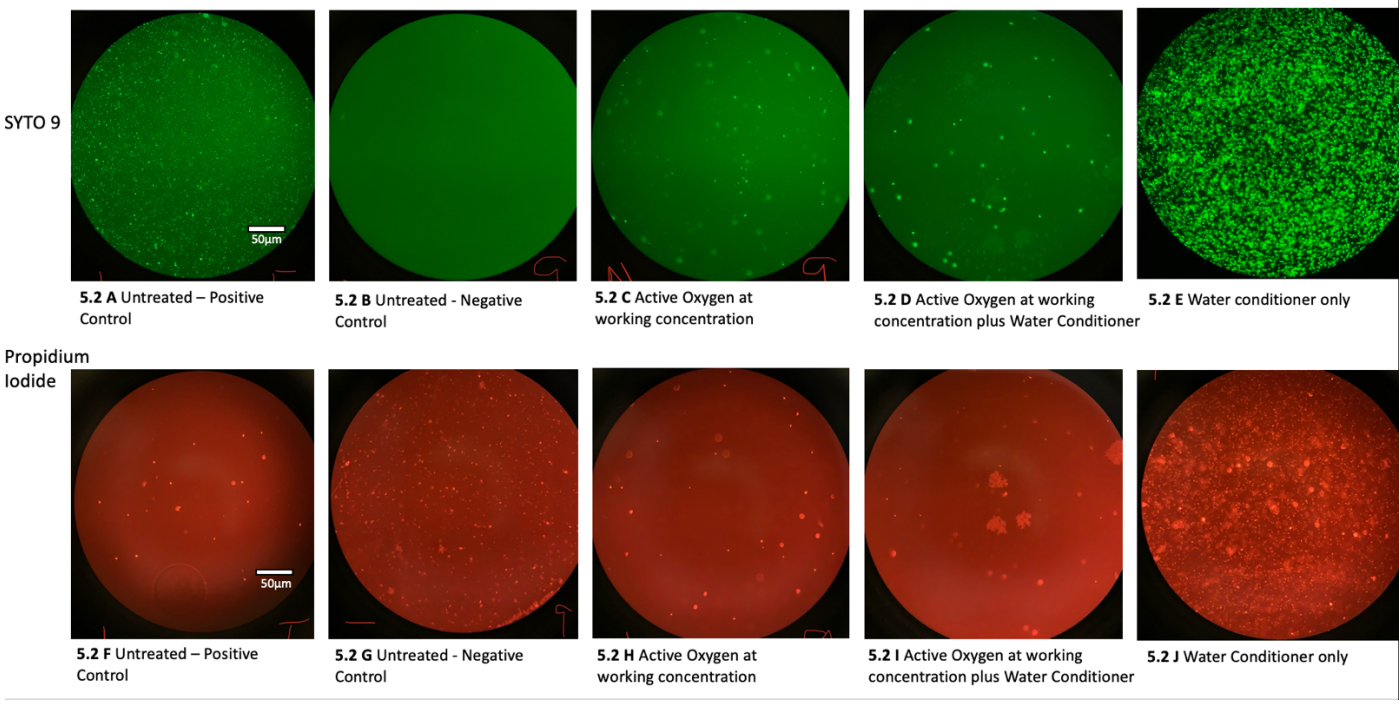


Figure 5.2. Effect of active oxygen and water conditioner alone or in combination on viable state of planktonic cells of *E. coli* K12 grown for 24h in M9 pH7.4 at 37°C. Live, viable cells are indicated by green-fluorescing cells that have taken up SYTO-9 (upper panels), cells assumed dead or with impaired membranes are indicated by red-fluorescing cells that have taken up propidium iodide (PI) (lower panels).

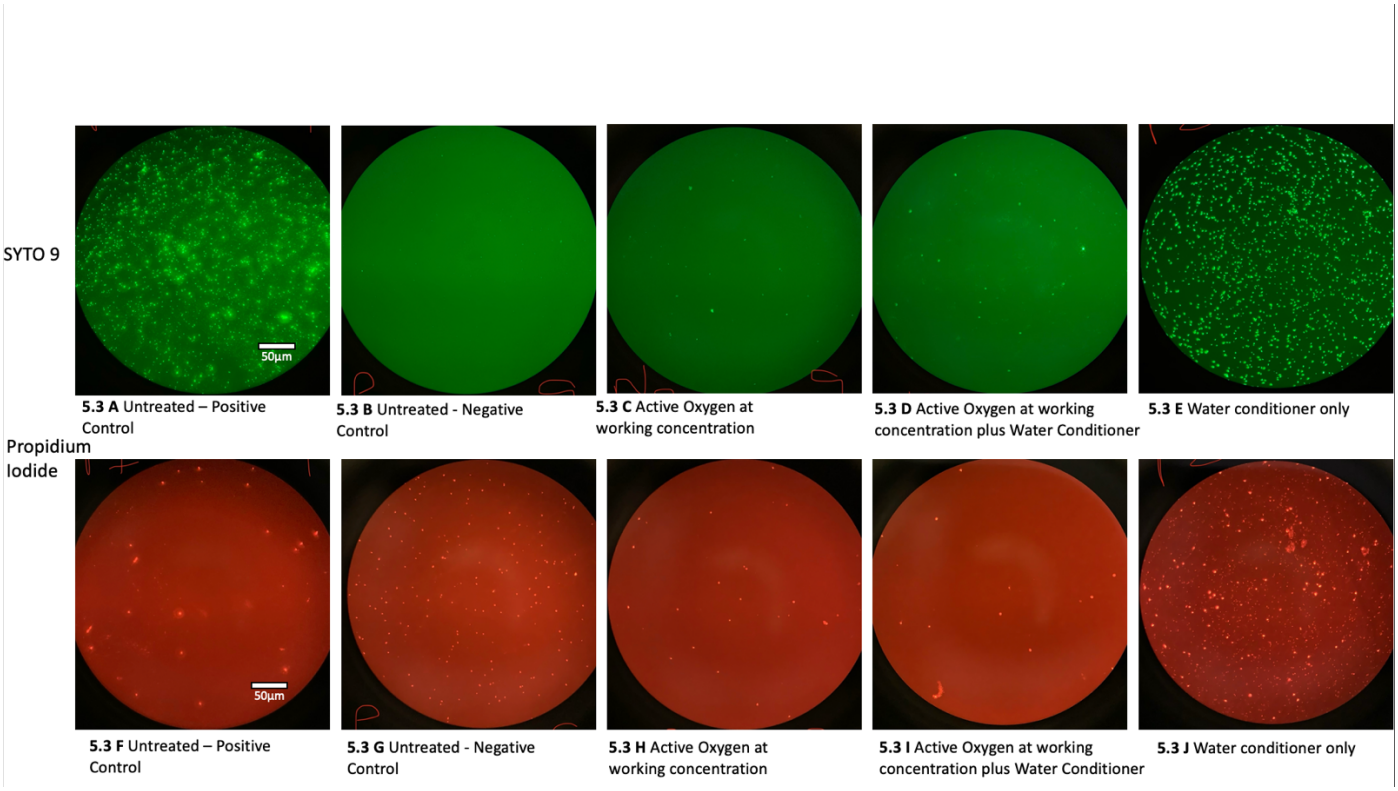


Figure 5.3. Effect of active oxygen and water conditioner alone or in combination on viable state of planktonic cells of *P. aeruginosa* PA01 grown for 24h in M9 pH7.4 at 37°C. Live, viable cells are indicated by green-fluorescing cells that have taken up SYTO-9 (upper panels), cells assumed dead or with impaired membranes are indicated by red-fluorescing cells that have taken up propidium iodide (PI) (lower panels).

Figures 5.2 and 5.3 show the effects of Active Oxygen by itself or in combination with the Water Conditioner on the viable state of *E. coli* K12 and *P. aeruginosa* PAO1 grown at 37°C at pH 7.4 for 24hrs, respectively. Throughout all light microscopy images, a high cell density can be observed. This is because the samples used were undiluted. Regarding live-dead microscopy, in the untreated control experiments, both species grew well thereby exhibiting a large number of live cells (as indicated by green-fluorescing cells) and a small number of dead cells (as indicated by red-fluorescing cells) (Fig. 5.2 A and F and 5.3 A and F). In cells treated with a known disinfectant (chlorine at 5 ppm) (5.2 B and G and 5.3 B and G), almost all cells were dead (red cells) and very few, if any live (green) cells were noted. Surprisingly, live (green) cells were noted for both species after treatment with Active Oxygen at normal operating concentration (Fig. 5.2 C and 5.3 C), although fewer *P. aeruginosa* cells were noted overall (Fig. 5.3 C and H). Interestingly, some clump-like structures (Fig. 5.2 H and 5.3 H) were

noted, but it was uncertain if they were morphologically similar to those seen in the previous light microscopy experiment (See section 5.2). In addition, there is a chance the cell clumping may have prevented the dyes from penetrating the cells. The addition of Product 2 by itself (Fig. 5.2 E and 5.3 E) also appeared to cause formation of small clumps. When treated with product 2 by itself, larger numbers of cells can be observed, however this may be due to clumping or unstable photo capture. Importantly, the combination of active oxygen with product 2 once again appears to form small chain-like or round clumps.

Overall, the number of live (green) cells found after treatment with known disinfectants was surprising as more dead (red) cells were expected. It would be worthwhile therefore to repeat fluorescence microscopy experiments to determine the viable state of cells to ensure the current observations are valid and not accidental.

5.4 Cell viability assays and efficacy of compounds on loss of cell viability

5.4.1 Efficacy of compounds on loss of cell viability - CFU data

In light of the observations made in section 5.3 which could not be repeated due to time and laboratory constraints, it was decided to determine cell viability following 24h treatments (Figs 4.1 A, 4.2 A, 4.9 A and 4.10 A), with active oxygen by counting the formation of colonies resulting from 16 hr incubation of serial dilutions of planktonic cell cultures which were then plated onto LB agar.

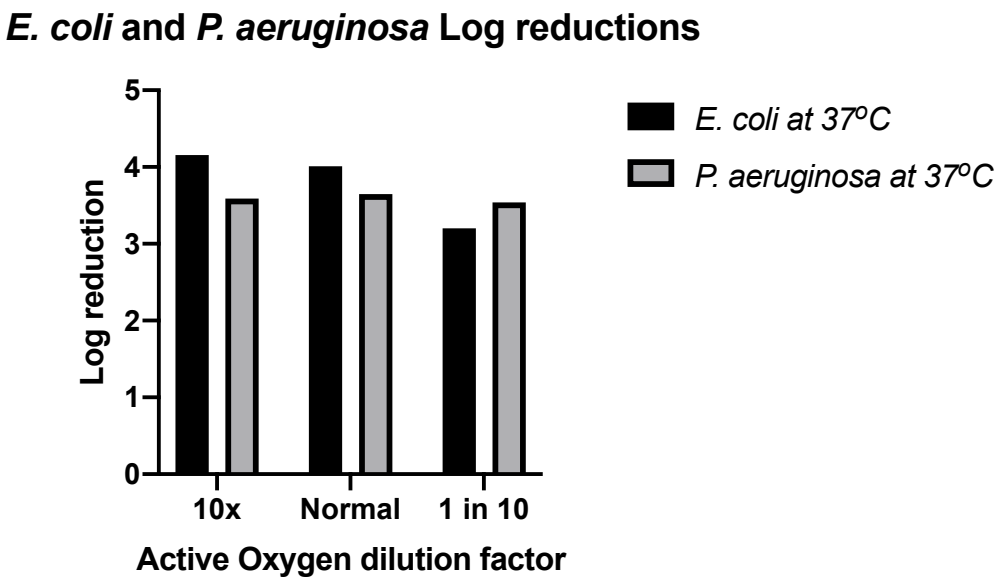


Figure 5.4: Loss of cell viability (shown as log reduction after CFU counting) of *E. coli* K12 and *P. aeruginosa* PAO1 following treatment with active oxygen. 'Normal' indicates usage of active oxygen at SOP, other concentrations as indicated relate to normal SOP. N=1 for biological repeats.

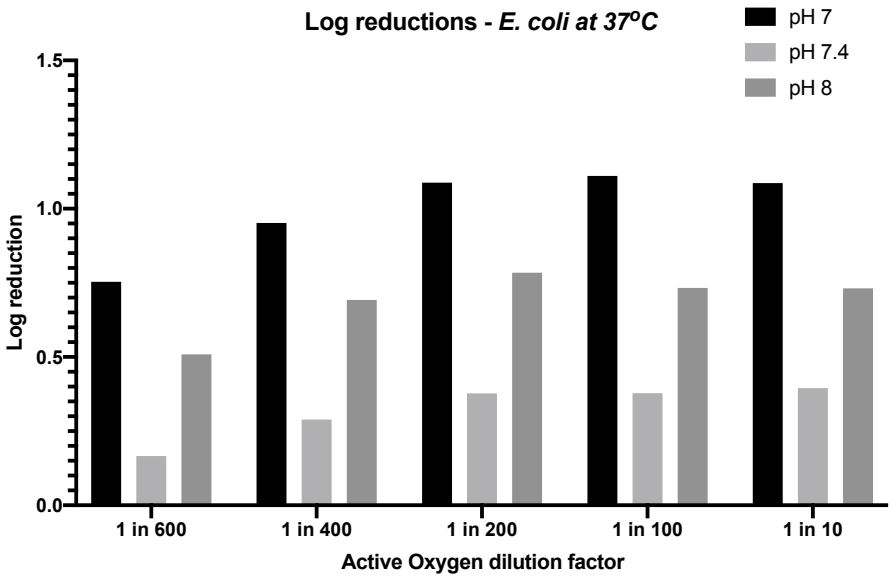
To calculate Log reductions from CFUs, the latter representing viable cells after treatment, samples were pipetted straight from the 96-well plates used in the SOP experiments in M9 media described in the previous chapter (Figs 4.1 A, 4.2 A, 4.9 A and 4.10 A), diluted serially and then plated on agar to allow counting of colonies resulting from cells that retained viability or were awoken from VNBC state. Samples were taken from *E. coli* and *P. aeruginosa* grown at 37°C, pH 7.4. Overall, a Log reduction of 4.11 was noted after *E. coli* treatment with 10x concentrated Active Oxygen, followed by a 4-Log reduction at the Normal concentration and a 3.2-Log reduction at one-tenth strength of active oxygen. *P. aeruginosa* showed slightly lower Log reductions, which proved less dose-dependent on active oxygen, though more consistent throughout the treatments. Treatment of *P. aeruginosa* led to the highest Log reduction at the Normal concentration at 3.64, followed by a 3.58-Log reduction at the 10x concentration and a 3.53-Log reduction at the 1:10 dilution.

Interestingly, the undiluted samples from cultures containing Active Oxygen and Product 2 (Water Conditioner) led to no CFU formation at all. Samples were also taken from cultures treated with chlorine at both concentrations (3ppm and 5ppm) and pH (7, 7.4 and 8). CFUs

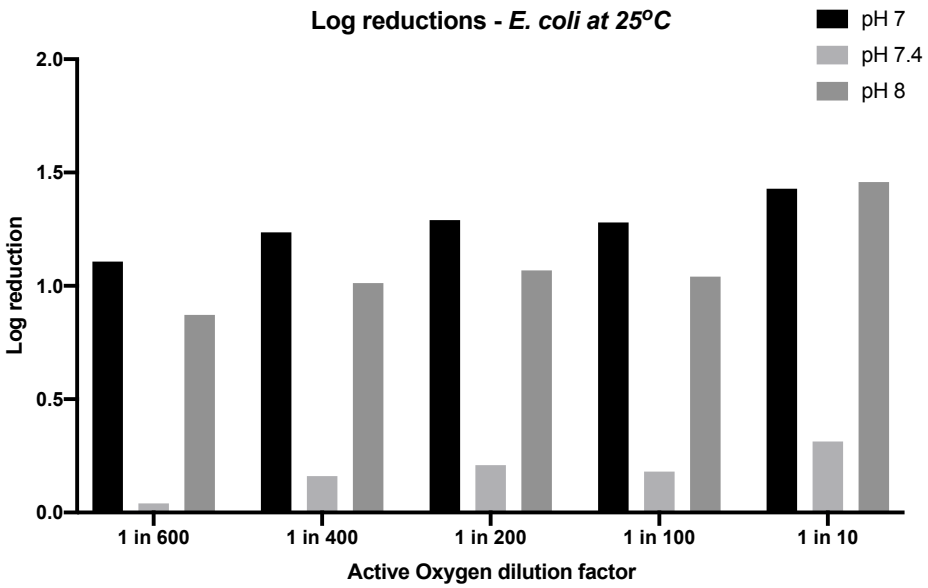
could also not be detected across these conditions. Rather than concluding these treatments were superior in water sanitation, the lack of any CFU formation is more suggestive of an accidental experimental error. Therefore, these experiments should be repeated before drawing final conclusions about efficacy of the combination or of the benchmarking product.

5.4.2 Efficacy of active oxygen on loss of cell viability – calibration curve derived data

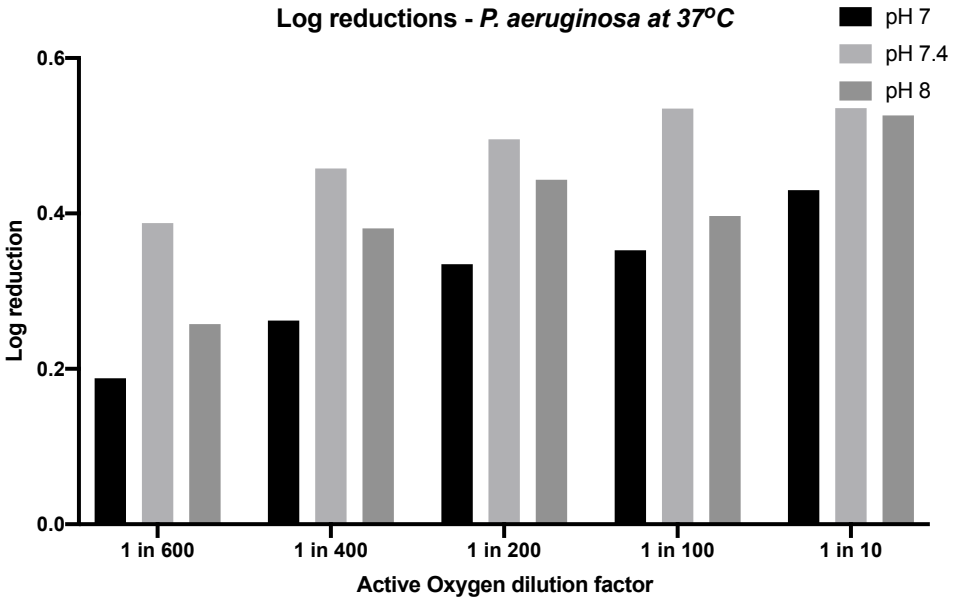
Due to time and laboratory access constraints, it was not possible to determine CFU-derived cell viabilities for all product treatments, and at all temperatures and pH ranges tested. Nonetheless, Log reductions could be derived by using cell density data obtained in the SOP experiments (Figs 4.1 A, 4.2 A, 4.9 A and 4.10 A) instead of pipetting samples on agar plates, which were converted to CFUs and then log reductions were calculated using OD-CFU calibration curves generated earlier for *E. coli* and *P. aeruginosa* (see section 3.2.5). Results with OD values below 0.1 and higher than 1 were excluded as the range of ODs used in the calibration curves were between 0.1 and 1. As results for treatments with Active Oxygen concentrations higher than one-tenth strength resulted in very low OD measurements, they were excluded from these derivations.



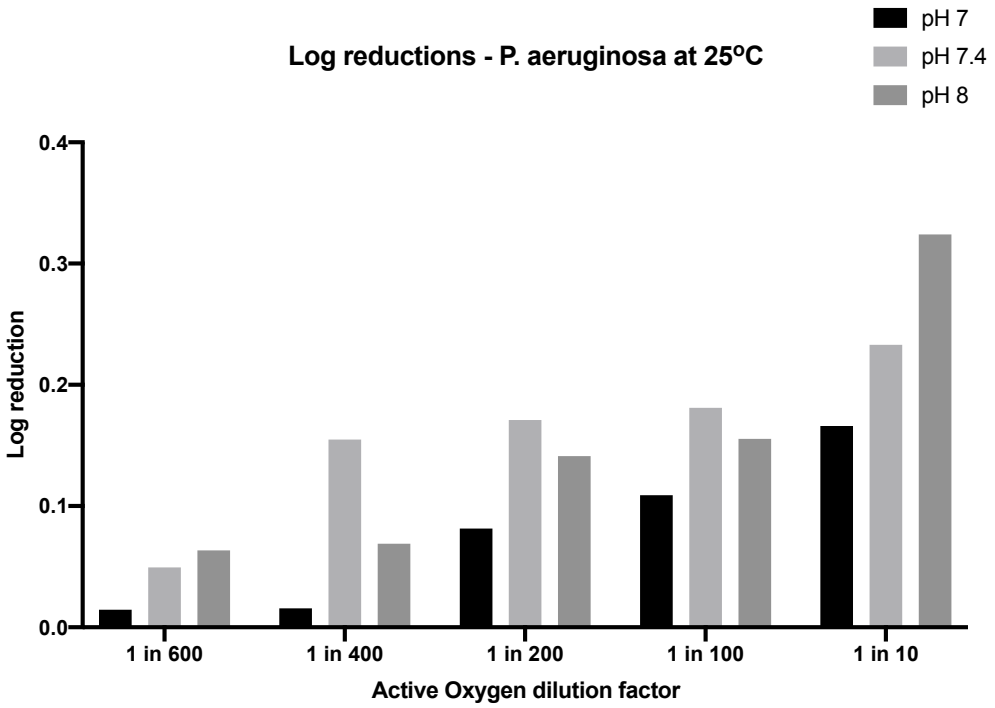
A


Log reductions - E. coli at 25°C
pH 7
pH 7.4
pH 8
Log reduction
2.0
1.5
1.0
0.5
0.0
1 in 600
1 in 400
1 in 200
1 in 100
1 in 10
Active Oxygen dilution factor

B


Log reductions - P. aeruginosa at 37°C
pH 7
pH 7.4
pH 8
Log reduction
0.6
0.4
0.2
0.0
1 in 600
1 in 400
1 in 200
1 in 100
1 in 10
Active Oxygen dilution factor

C



D

Figures 5.5 A-D Cell viabilities (shown as log reductions derived from OD595-CFU conversions) following treatment with active oxygen at sub-SOP concentrations for *E. coli* (A-B) and *P. aeruginosa* (C-D) grown in M9 at 37°C (A and C) and 25°C (B and D) at different pHs.

Overall, low log reductions were noted as expected due to relatively good planktonic growth. The highest derived Log reductions overall were noted for *E. coli* at 37ºC, with Active Oxygen in concentrations stronger than 1:200 at pH 7 resulting in a Log reduction higher than 1, followed by Active Oxygen at pH 8, with Log reductions noted in the range between 0.5 and 0.75. Regarding *E. coli* at 25ºC, all Active Oxygen dilutions at pH 7 resulted in a Log reduction higher than 1, followed by Active Oxygen at pH 8 with similar results. The highest Log reductions for *P. aeruginosa* at both temperatures were noted at pH 7.4, followed by pH 8. Log reductions for *P. aeruginosa* are lower than *E. coli*. In addition, the highest log reduction for *P. aeruginosa* at 25ºC was noted at pH 8.

5.5 Discussion

Light microscopy showed high cell numbers in untreated wells, indicating that the growth conditions were optimal for *E. coli.* Active oxygen treatment in all concentrations showed the presence of motile cells. The addition of water conditioner (Product 2) in combination with Active Oxygen resulted in a decrease in cell motility and the formation of cell clumps. Live-dead staining showed high numbers of live cells and low numbers of dead cells in the control wells for both species, indicating optimal growth conditions. Live cells were noted for both species after treatment with Active Oxygen at SOP concentration and some small clumps were noted after the addition of water conditioner. Viability assays after active oxygen treatment at three concentrations showed a Log reduction higher than 3.5 for both species. The addition of Product 2 (conditioner) led to no visible CFUs. No CFUs were also noted after chlorine treatment. Calibration curve derived data showed lower levels of reduction; however, they were dependent on the difference between control and treated wells, which was not large in some cases. From the experimental results and with limitations, it can be concluded that active oxygen achieves a log reduction higher than 3.5, the combination with water conditioner causes clumping and the development of viable cells but non-culturable on agar, and water conditioner by itself does not appear to have a significant bactericidal effect. Although live-dead staining showed some challenges, it ended up providing crucial insights on cell morphology and clumping.

Interestingly, the undiluted samples from the wells containing Active Oxygen and Product 2 (Water Conditioner) led to no CFU formation at all. This is interesting, as samples from the same wells used for fluorescence microscopy (Fig. 5.9 A-B and 5.14 A-B) showed that there were live cells present. If the lack of CFU formation were not an accidental experimental error, then this could potentially be an exciting observation. This as it may suggest that the product combination may lead to the formation of viable but non-culturable cells (VBNC). VNBC cells are defined as living cells which, however, cannot grow on agar, unable to produce colony forming units and is of great importance in research as it may lead to underestimation of the number of cells present in a sample during CFU counting, a standard microbiology technique (Li et al., 2014). *E. coli* and *P. aeruginosa* have both been shown capability to enter VBNC state (Zhang et al., 2015). Characteristics of VBNC cells include intact cell membranes in contrast to dead cells with ruptured membranes, plasmid retention, active cellular metabolism and respiration and active transcription of genes, plus uptake of nutrients (Li et al., 2014).

However, what makes VBNC cells potentially dangerous is the fact that although they may be avirulent in their current state, upon entering favorable condition they could resuscitate into virulent culturable cells (Du et al., 2007). Therefore, it would also be of interest to see whether the cells in this project can resuscitate in hot tub operational conditions.

RpoS, whose role in the stress response has been explained extensively in this thesis, unsurprisingly plays a role in VBNC formation in *E. coli,* with studies showing that RpoS helps cells stay in the VBNC state for longer, with mutants exiting the phase sooner and dying. The same study found that RpoS is responsible for maintaining cells culturable, with RpoS mutants losing their cultivability faster (Boaretti et al., 2003).

Regarding 'live-dead' cell staining, one critical consideration is that the fluorescent dyes used depend on the cells' membrane integrity. It is assumed that viable cells have intact cell membranes that do not allow 'dead' stains to pass through, while 'dead' cells have ruptured cell membranes (Stiefel et al., 2015). SYTO 9 and Propidium iodide, the fluorescent stains used in this project, also work based on this assumption (Stiefel et al., 2015). As VBNC cells have intact cell membranes, it makes sense that they would be stained green and thought of as live cells (Stiefel et al., 2015). This can provide an explanation to the phenomenon observed in this project, where Active Oxygen in combination with Product 2 could potentially lead to VBNC formation and is an interesting product combination phenotype to note.

Chapter 6: Review of protein components in Product 2 (Water conditioner)

6.1 Introduction

Results described in Chapters 4 and 5 indicate that (combinations of) treatments with eco3spa Product 2 (Water conditioner) at standard operating concentration yielded interesting observations regarding overall product efficacy as well as cell morphology and viability. The combination of the water conditioner with active oxygen did not inhibit the activity of active oxygen and may even have supported it (section 4.2.6 -4.2.7). Additionally, the addition of the water conditioner by itself appears to have a small biofilm inhibitory effect (section 4.2.6-4.2.7). The combination of active oxygen and water conditioner caused the appearance of clump-like cell aggregates (section 5.3), while possibly also changing the viable status and/or membrane integrity of treated cells (section 5.3).

The content listing of Product 2 details a blend of glycosides, a tertiary amine oxide, an isothiazolinones preservative, salt, citric acid, a blend of coconut fatty acids, and a mixture of proteins with hydrolytic activities: lipase, protease and amylase in a composition of 90% lipase, 5% protease and 5% amylase. Due to the interesting phenotypes when cells were exposed to Product 2, it is of interest to test each component separately to determine the source of the activity of whether it is the results of an interaction with other components in Product 2 and/or Active Oxygen.

Due to time and laboratory constraints, it was not possible to investigate each component in Product 2 in this project. It was decided to prioritize a very brief investigation of protein concentration and activity of one quickly assessed enzyme (amylase), benchmarked against commercially available enzymes due to ease of access to bioanalytical instrumentation and immediate availability of bio-chemicals.

The aim of this chapter therefore was to assess the overall protein concentration and determine amylase activity in eco3spa Product 2.

6.2 Results

6.2.1 Protein standard graph

Bovine serum albumin (BSA) was used as standard to generate a protein calibration curve (Fig 6.1). The resulting trendline equation was used to calculate the protein content of the

commercial enzyme preparations (amylase, lipase and protease solutions) in addition to the water conditioner product. The protein concentration of the samples is shown in Table 6.1.

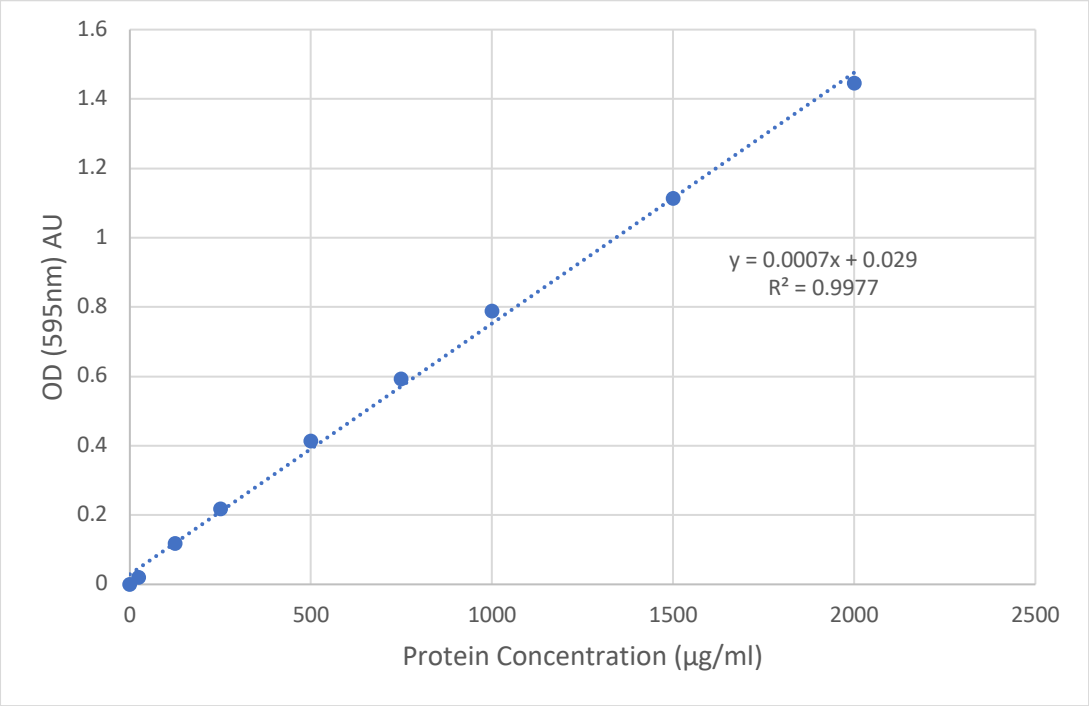


Figure 6.1- Protein standard curve plotting OD595 against known BSA protein concentrations.

A series of diluted albumin (BSA) standards covering a range of 25 to 2000 µg/mL were prepared according to the manufacturer's instructions and incubated at 37°C for 30 mins. The protein concentration (µg/ml) indicated that the suitable protein gel would be a Bis-Tris gel utilizing the MOPS buffer (Refer to table 6.1).

Table 6.1. Protein concentration of enzymes and water conditioner

Sample	Protein concentration (µg/ml)
Amylase	38.787
Protease	25.330
Lipase	6.9511
Water conditioner	1.2705

6.2.2 Visualization of enzyme and water conditioner proteins

It was decided to also conduct protein electrophoresis to enable visualization of any protein present in the Product 2, which would also allow size investigation and comparison of the proteins against the commercial preparations of single enzymes. Figure 6.2 shows the result of protein resolved on NuPAGE 10%, Bis-Tris gels. The commercial enzymes were resolved, indicating a single protein of 55 kDa in the amylase sample (Fig 6.2 lane B), two protein bands of sizes 60 and 38 kDa for the protease sample (Fig 6.2 lane C), two main protein bands of sizes 55 and 32 kDa, which were accompanied further by minor protein bands of sizes 28 and 24 kDa for the lipase sample (Fig 6.2 lane D). Protein band(s) could alas not be successfully detected for Product 2 (Fig 6.2 lane E, indicated by the red-boxed area. According to total protein analysis, the water conditioner should have contained 1.27 ug of protein, which Coomassie stain would have been able to visualize. This experiment should therefore be repeated to confirm the presence of any protein in Product 2.

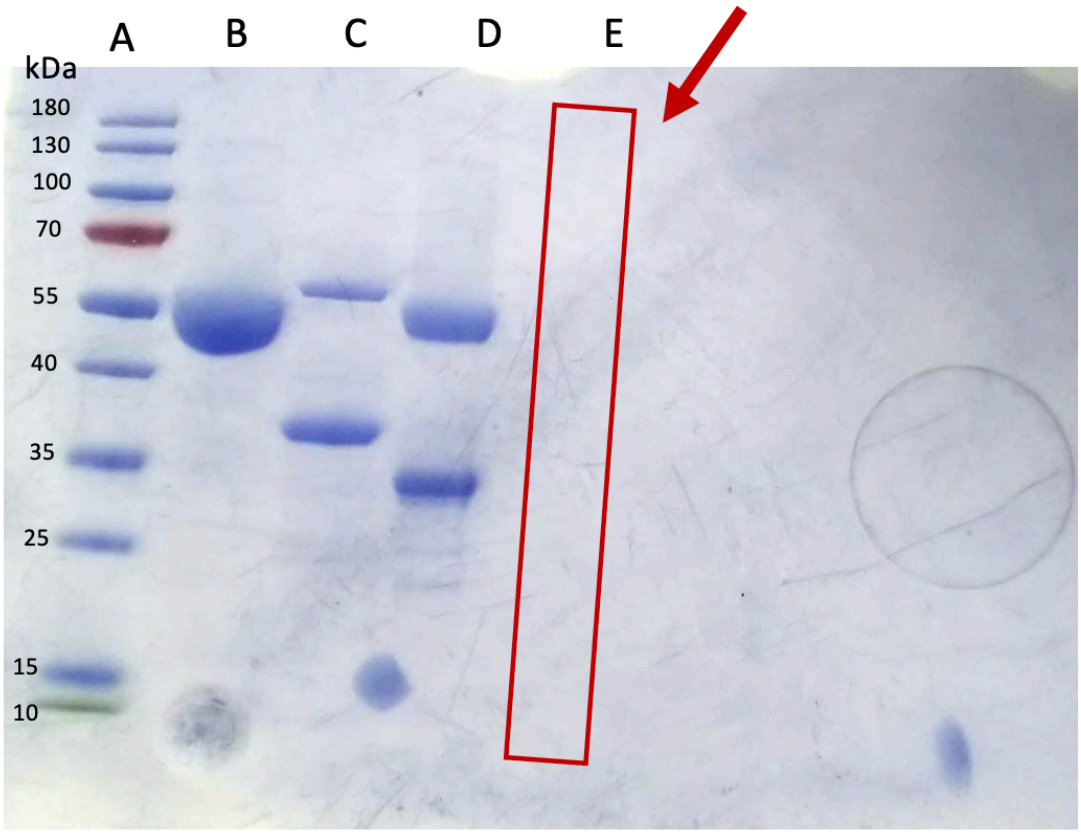


Figure 6.2. Comparison of Product 2 with commercial enzyme preparations resolved on Coomassie-stained NuPage 10% Bis-Tris gel. A, Pre-stained protein standards; B, 38.78 µg amylase; C, 25.3 µg protease; D, 6.95 µg lipase; E, 6µL µl Water Conditioner (supposedly containing 1.27 µg protein).

6.2.3 Protein concentration efforts

Surprisingly, according to the protein gel results, lane E showed no bands which would suggest the lack of enzymes present or at a level that is undetectable by Coomassie blue. To further assess whether any protein was present in the water conditioner and remove any interfering substances, such as oils, a TCA precipitation procedure and an acetone precipitation procedure were performed, a pellet was formed, and the extracted product was used in a BSA assay to check whether any protein was present, but no protein was detected, or the levels were below assay threshold.

6.2.4 Lugol iodine assay

As protein electrophoresis failed to detect any protein in Product 2 and due to time restraint, it was decided to perform a more sensitive protein activity experiment. The amylase enzyme assay was selected for it is non-complicated and rapid, for which all assay components were available, in order to check whether any active amylase was present in the Water Conditioner at all. A 1% soluble starch solution was prepared as substrate, which was then mixed with the commercial amylase solution as positive control, or with Product 2. Following incubation at room temperature at different time intervals, amylase activity was then visualized by adding Lugol-iodine solution. Lugol-iodine stains dark blue/black when starch is present, while it is amber-red in the absence of starch. Figure 6.3 shows the results of one amylase activity assay (N=1).

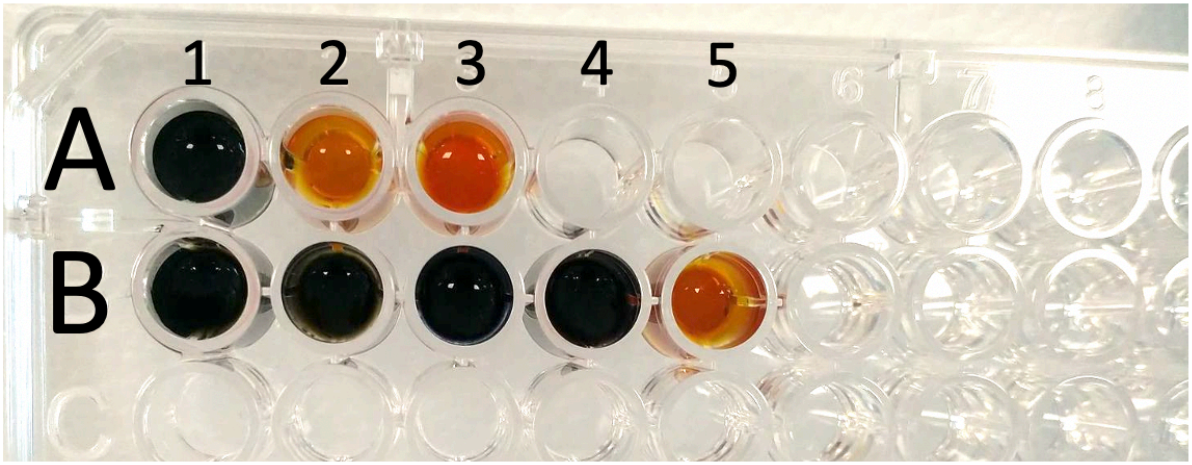


Figure 6.3- Qualitative assay for amylase activity in control and Product 2 using a Lugol iodine assay. A1, starch (negative control); A2 and B5, starch and commercial amylase (positive control); A3 commercial amylase (negative control); B1, starch plus Product 2 upon mixing (0h); B2, starch plus Product 2 after 1h incubation; B3, starch plus Product 2 after 16h incubation; B4, Product 2. Lugol-iodine was added to stain starch.

The combination of starch and iodine led to the immediate development of an almost-black color, while the immediate addition of amylase and starch into iodine led to the development of a pale amber color. Amylase by itself led to the development of a darker amber color. Product 2 (water conditioner) was mixed with starch and incubated for a range of 0-16 hrs. and samples were pipetted into iodine. Regardless of incubation time, no color change was observed. Interestingly, the addition of water conditioner also led to positive staining, which could either result from the listed glycosides, or perhaps result from other components. Regardless, this experiment should be repeated to confirm the presence or absence of amylase in Product 2

6.3 Discussion

Due to experimental limitations and the unanswered question regarding the protein composition of the water conditioner, a Lugol iodine assay was performed to qualitatively assess if any amylase was present in the water conditioner. Lugol is commonly used to detect starch and polysaccharides (Sehnke et al., 2001). However, at no point during the incubation period was a color change detected, even after overnight incubation, when Product 2 was added. The fact that Lugol changed color in reaction to the Water Conditioner by itself indicates that the product indeed contains glycosides, as the ingredients list mentioned. It is surprising that no color change to amber was noted at any point, which suggests that no active amylase is present, unlike the product description. Precipitation assays and protein visualizations also indicated that no protein is present at all.

The effects observed after the addition of Water Conditioner together with Active Oxygen such as cell clumping and conversion of viable cells to VBNC are therefore difficult to attribute to amylase, as no protein content was detected, assuming these initial findings need to be re-analyzed in repeat experiments.

It is also possible that another component of the Water Conditioner could be responsible for the possible enhancement of Active Oxygen activity, possibly a pH regulator as mentioned before, the coconut fatty acid or another component yet to be positively linked conclusively to the observed phenotypes.

Chapter 7: General Discussion, Reflections and Conclusions

7.1 Introduction

This project involved eco3Spa, specialty and eco-friendly hot tub sanitation product distributors for the wet leisure industry in the UK. They provide products designed for water sanitation, and biofilm prevention and removal in hot tubs. However, prior to this project Eco3Spa did not possess the scientific underpinning of efficacy and the effects of the product and/or its product combinations, as well as the effect of pH and temperature on product efficacy. Eco3Spa also wanted high-quality, ready to publish graphs for marketing and promotion purposes, as well as information on the number of bacteria present in samples and Log reduction data.

This project addressed the question of whether eco3spa products are able to inhibit planktonic growth and/or reduce/remove biofilm under static conditions at the recommended and other concentrations. This has been achieved through the optimization of planktonic growth and biofilm assays that allowed levels of planktonic bacterial growth and biofilm formation in the presence or absence of these products to be quantified accurately. Furthermore, the results have been interpreted in the context of published literature on biofilm properties, bacterial growth, stress response and bacterial adaptation to environmental conditions, which has given insight into how the product(s) may act.

7.2 Optimization of studies

A large part of the research project was dedicated to estimating, detecting and optimizing the experimental conditions in order to determine the exact conditions that not only produce replicable results, but also simulate the hot tub environment, leading to the development of Chapter 3 - Optimization and Chapter 4 – non SOP experiments. This chapter allowed a biofilm assay to be designed that mimicked the conditions found within a hot tub or swim spa setting. The bacteria selected (*E. coli* and *P. aeruginosa)* for testing were strains commonly found in hot tubs, which can be responsible for development of disease in the elderly and immunocompromised people (Fewtrell and Kay, 2015; Lutz and Lee, 2011). *P. aeruginosa* was of particular interest, as it is not only commonly found in recreational waters but is also notorious for not only forming persistent biofilms in the aquatic environment, but also for

being resistant to antibiotics and disinfectants, making its prevention and removal a challenge (Lutz and Lee, 2011; Fontes et al., 2012).

It was also important to consider the conditions found within a hot tub environment. Two incubation temperatures of 25 and 37 degrees were used for biofilm incubation, to represent the water temperature when the hot tub was switched off and the normal temperature of a hot tub during use (Hot tub manual, 2019). In addition, the media selected also had to reflect the different conditions under which biofilm may grow. Although some experiments were undertaken in LB media, because of time constraints, it was decided to prioritize use of M9 as the main medium, to simulate the nutrient-poor environment of the hot tub water. The sponsoring company was in agreement with this decision (Hammond et al., 2010; Naves et al., 2008).

Finally, it was also critical to optimize the experimental conditions to ensure accuracy and precision. For example, the extend of the edge effect was assessed, along with the number of washes, washing materials, drying and staining times, preparation of Active Oxygen and chlorine etc. Moreover, information was also gathered regarding incubation temperatures and times, differences in media regarding planktonic growth and biofilm formation and the effect of media in tandem with incubation time on the experiments, which not only assisted with the experiments of this project but may also provide insights to future research.

7.3 Effect of temperature

In M9 media SOP experiments, untreated *E. coli* exhibited higher levels of planktonic growth in comparison with untreated *P. aeruginosa,* where planktonic growth remained in lower levels (Figs. 4.1 A-B, 4.2 A-B). In LB experiments, *E. coli* reached higher planktonic growth in comparison with *P. aeruginosa* (4.13 and 4.14). Regarding biofilm formation, in M9 media and SOP experiments, *P. aeruginosa* showed the highest levels of biofilm formation at 37°C in comparison to 25°C (Fig 4.8). Similarly, *E. coli* showed higher biofilm formation in the same experiment at 37°C (Fig. 4.8). Regarding growth in LB media after 48hrs, the highest biofilm formation noted across all experiment was produced by *P. aeruginosa* at 37°C. It can be concluded that the highest planktonic growth and biofilm formation overall were noted at

37°C and that higher temperature influences growth and biofilm formation of *P. aeruginosa* at a greater degree than *E. coli.*

Static biofilm assays showed that the Active Oxygen tablets, in SOP conditions, are effective at preventing planktonic growth and biofilm formation for both *E. coli* and *P. aeruginosa.* For biofilm prevention, no biofilm was quantified at all after treatment with the SOP concentration, 10x concentration as well as the 1 in 2 SOP concentration. Planktonic growth and biofilm formation would start forming at relatively low levels in the 1 in 10 concentration and in concentrations lower than this, the product would begin gradually losing its efficacy. The product exhibited similar planktonic growth and biofilm prevention properties in both strains assessed, with slightly lower efficacy noted at biofilm prevention at pH 8 and 25^{o}C. It can be hypothesized that temperature may play a role in enhancing the activity of Active Oxygen, and it would be interesting to assess the effect of temperature cycling between 25 and 37 degrees to simulate the hot tub environment. It is also hypothesized that high temperature may play a role in the activation of the tablets, as the product description indicates dissolving the tablets in a functioning hot tub with the temperature of water set up at around 37°C, and there are large differences in active oxygen activity between SOP and non-SOP experiments.

Regarding non-SOP experiments, although the active oxygen tablets are intended as a preventative treatment, they still had an effect in inhibiting planktonic growth and a bactericidal effect, even after 24hrs incubation, especially at the 10x concentration, which is more pronounced at 37°C (Fig 4.2 A-D). In addition, the water conditioner and biofilm remover, even though they are not intended for killing bacteria, also had a similar effect which was also enhanced by high temperature (Fig 4.2 B-D). Similar trends can be observed throughout most experiments in this project. It can therefore be concluded that high temperature is indeed important for the optimal activity of products tested and may play a role in proper dissolution, activation, or enhancement.

7.4 Effect of pH

In M9 media SOP experiments, the effect of pH on planktonic growth, biofilm formation and active oxygen activity was assessed. At pH 7, *E. coli* showed the highest planktonic growth overall (Fig, 4.7 A). At pH 7.4, *E. coli* and *P. aeruginosa* at 37°C surpassed bacteria at 25°C (Fig, 4.7 B), and at pH 8, both strains showed similarly high levels of planktonic growth with the exception of *P. aeruginosa* at 25°C (Fig. 4.7 C). pH is also important in biofilm formation. Untreated *E. coli* showed the highest biofilm formation at pH 7.4 for both 25^{o}C and 37^{o}C (Fig. 4.3 A and B). At high pH values, *E. coli* showed very low biofilm formation (Fig. 4.8 C), in contrast with *P. aeruginosa* which showed biofilm formation at higher pH, especially at pH. 8 (Fig. 4.8 C). It can be concluded that the highest planktonic growth and biofilm formation for *E. coli* were noted at low pH, in contrast with *P. aeruginosa* which proliferated at high pH.

In general, the Active Oxygen product is effective at reducing planktonic growth across the range of pH tested. Both *E. coli* and *P aeruginosa* showed low levels of planktonic growth after active oxygen treatment. However, some loss of activity was noted for both species at 25^{o}C and in particular at pH 8 (4.1 B and 4.2 B). In addition, regarding biofilm formation, very low levels were detected after treating *E. coli,* even with active oxygen concentrations higher than 1 in 10 SOP. It is possible that the small amounts of planktonic growth noted for both species at 25^{o}C, and in particular at pH 8 (4.1 B and 4.2 B), may be at the viable-but-not-culturable state and may not produce biofilm (Li et al., 2014).The European Chemicals Agency has indicated that the form of active oxygen present in the tablets (pentapotassium peroxymonosulfate) is degraded at low temperatures and high pH. Therefore, the loss of activity noted at low temperature (25^{o}C) and high pH makes sense. It would also be interesting to use a wider pH range to further investigate the effect of pH on Active Oxygen. As the Active Oxygen prevented biofilm growth in the SOP experiments, the product can be considered as effective at preventing biofilm.

In addition, several studies have indicated that biofilm removal products containing levels of biocidal chemicals that fall below the European Chemicals Agencies can enhance planktonic growth of bacteria by inducing stress which can enhance biofilm formation (Battesti et al.,

2011). This was indeed observed in this project (Fig. 4.4 A), where low pH and suboptimal MIC led to increased biofilm formation. This is a well-known effect, and several antimicrobials and disinfectants at sub-optimal MIC have shown to induce biofilm formation (Majtan et al., 2008).

7.5 Benchmarking of product against chlorine

Regarding the benchmarking of chlorine against active oxygen, the two concentrations recommended to be included in experiments by the company (3ppm and 5ppm) were the two concentrations used in hot tubs and swim spas. Chlorine did not show a significant loss of activity at either pH 7 or pH 8, however in comparison with Active Oxygen at the normal dilution, Active Oxygen exhibited higher stability and statistically significant higher sanitizing activity. Moreover, active oxygen exhibited stable sanitation activity across all pH tested, even at pH 8. The knowledge that tap water in some regions of the UK has been found to be in the basic range which degrades chorine and the fact that sunlight further degrades chlorine, make active oxygen an attractive alternative(Ander et al., 2016)Jain et al., 2010).

Samples from bacteria treated with chlorine at either 3ppm or 5ppm in all pH did not lead to CFU formation. This suggests that chlorine treatment may lead to VBNC formation, and the small levels of cells responsible for the planktonic growth noted during benchmarking may be incapable of forming a biofilm.

7.6 Active Oxygen dose-response

The dose response curves generated provided valuable insights on the activity of active oxygen in a series of dilutions against both species under a range of temperatures and pH. Both species showed sensitivity even to the lowest active oxygen concentration (1 in 600 SOP concentration), which suggests that active oxygen is capable of reducing planktonic growth even in low concentrations. The 1 in 10 SOP concentration in M9 media was the lowest tested concentration capable of preventing planktonic growth to very low levels in both species, and concentrations higher than that were equally effective (Section 4.2.1).

Similar results were noted for biofilm formation. Once more, both species showed sensitivity even to the lowest active oxygen concentration (1 in 600 SOP concentration), which suggests that active oxygen is capable of preventing biofilm formation to some degree even in low concentrations. The 1 in 10 SOP concentration in M9 media was the lowest tested concentration capable of preventing biofilm formation to very low levels in both species, and concentrations higher than that were equally effective (Section 4.2.2).

Light microscopy showed live (motile) cells in after treatment with active oxygen in a series of concentrations, which was surprising. Furthermore, live/dead staining showed significant numbers of live cells in populations treated with a series of active oxygen concentrations, which raised questions regarding the bactericidal efficacy of the product. However, CFU viability assays generated very few colony-forming cells. In addition, the assays showed the product was capable of achieving an average 3.5 log reduction in both species, which confirmed its sanitizing efficacy. The results from the viability assays and the absence or presence of very few of colony forming units led to the speculation that active oxygen may lead to formation of VBNC cells. The very low levels of planktonic growth were noted for both species at 25°C at the concentrations mentioned, however as no biofilm formation was noted in by the cells under those treatments, in combination with the microscopy results may further hint at VBNC formation.

7.7 Efficacy of products alone and in combination

The addition of Product 2 (Water conditioner) in combination with active oxygen again did not appear to either assist not inhibit active oxygen activity (Sections 4.2.6, 4.2.7). In addition, pH did not appear to play a role in the efficacy of the combinations. The addition of Product 2 by itself did appear to have a small effect in preventing planktonic growth and biofilm formation, but whether this is caused by the conditioner or by nutrient deprivation is not clear and needs more investigation. Interestingly, a small increase in optical density and biofilm formation after treatment of cells in both species with active oxygen in the 1 in 10 concentration plus conditioner was seen. It could be hypothesized that in sub-optimal stress conditions the addition of Product 2 could cause stress-induced biofilm formation, which may have an effect on virulence and pathogenicity. In addition, it could be possible that some of

the components of the conditioner may be used as a nutrient source by the surviving bacteria, or that components may be interfering with the OD measurements.

In addition, the addition of product 1 (biofilm remover) also appeared to have a small effect in preventing planktonic growth and biofilm formation (Section 4.2.8). Surfactants, which are present in the product, have been shown to prevent biofilm formation in a range of bacteria, including *E. coli* and *P. aeruginosa* (Wu et al., 2013). However, as the project's main interest was on active oxygen, the biofilm remover was included in very few experiments and more investigation may reveal synergistic effects of the product with active oxygen or water conditioner.

Light microscopy provided some insights on the morphological changes induced by the product combinations. In particular, it was noted that the addition of the water conditioner in combination with active oxygen led to the formation of clump-like structures and immobile cells. This was further investigated using fluorescence microscopy and live/dead staining cells, which showed the formation of some clump-like structures. The addition of water conditioner by itself also appeared to cause clump formation, although it is not entirely clear, and a repeat of the experiment is needed. It should be noted that live-dead staining does have some drawbacks, such as depending on cell membrane integrity to determine viability, and alternative viability assessment methods may provide more insights on the topic. Moreover, CFU viability assays did not generate any colony-forming cells at all. The results from the viability assays and the absence of colony forming units led to the speculation that the combination of water conditioner with active oxygen may lead to formation of VBNC cells. However, more testing is needed to confirm this. In particular, the use of confocal microscopy or flow cell cytometry can be helpful in the determination of cell morphology and viability, as well as the use of dyes specific for enzymatic activity or respiration (Rosenberg et al., 2019). Another morphological change that may be observed in stressed bacteria is the formation of small colony variants (SCVs). SCVs are naturally occurring variants which provide cells with a survival advantage by protecting them from host defences and antibiotics, most commonly in the human body. They appear as non-pigmented, non-haemolytic colonies ~10 times smaller than those of the normal phenotype and can be recovered through the use of

nutritional supplementation (Garcia et al., 2013). Unfortunately, due to limited lab time and experimental limitations it was not possible to test for these variants.

The protein gel showed no bands in the lane where water conditioner was tested, and BCA assays together with precipitation assays showed that no protein was present in the product in detectable levels. In addition, a Lugol iodine assay indicated that no active amylase was present in the product, however significant levels of glycosides, which according to the manufacturer were present in the conditioner, or sugar-based molecules, may have reacted with the iodine. These results suggest that the enzymes were not responsible for the synergistic effect of the water conditioner with active oxygen. According to the sponsoring company, the product contains coconut fatty acids and citrate, which may act as a pH regulator, and it is possible that either one of these components or some other ingredient may be responsible for the synergistic effect. However, as the experiments found no evidence of enzymatic activity or protein presence, it would be worthwhile to confirm the presence of any potentially active ingredients.

7.8 List of conclusions

After performing a literature review, the experiments presented in this project and a critical appraisal of the results obtained, it can be concluded that the questions set at the beginning of the project, in agreement with the sponsoring company, have been answered. In particular, the conclusions reached in this project are:

- Active oxygen at SOP is effective at preventing planktonic growth and biofilm formation for both *E. coli* and *P. aeruginosa* in concentrations higher than 1 in 10 SOP, at both 25°C and 37°C and over a range of pH (Chapter 4)
- The addition of water conditioner appears to neither enhance not inhibit the sanitizing efficacy of active oxygen and may lead to generation of clumps and switch cells to VBNC (Chapter 4 and Chapter 5).
- Active oxygen achieves an average 3.5 Log reduction against both species (Chapter 5)

- The active oxygen tablets perform at least as well as chlorine in preventing planktonic growth and biofilm formation, and may even be more stable at extreme pH (Chapter 3)
- Constant pH balancing is not required when using the active oxygen tablets
- Attempts to biochemically analyze the water conditioner showed that no enzymes or protein were present.

7.9 Limitations

Because of the effect of COVID-19 on lab access, and a 6-month absence from laboratories, the experimental time of this project was cut short. However, despite these difficulties, this project has achieved the goals set out by the company and provided important insights on the sanitizing efficacy of active oxygen and the effects of product combinations on bacterial growth and biofilm formation.

Other than the very limited lab time available, many experiments were performed under time constraints, lab equipment limitations and under n=1 conditions. For example, upon return to laboratories in August for a 4-week period a rota system was in place that severely limited the available experimental time. Therefore, only SOP experiments and combinations prioritized by the company were performed, and only in M9 minimal media. Additionally, there was no time available to confirm or optimize any initial, non-SOP experiments.

In addition, experiments and experimental procedures performed for the first time, such as light and fluorescence microscopy and protein assays were performed under lab equipment limitations and under n=1 conditions. For example, regarding fluorescence microscopy, lack of computer-controlled image capture software led to suboptimal image capture via mobile phone camera.

Importantly, the project originally aimed to incorporate elements of chemical analysis which would have provided insights on the activity of active oxygen including the use of radical scavengers to measure the activity of Active Oxygen, and HACH assays to measure the activity and concentration of chlorine, which could potentially provide answers to some of the

questions generated in this project. As the estimation of chlorine was made using test strips and their color could be subjective, there is a chance of inaccuracies in chlorine concentrations, and a more accurate chlorine measurement system could solve this. However, because of the fire on the Bay Campus which severely damaged the chemical engineering labs, there was inability to access the necessary equipment.

Furthermore, because of lack of in-person courses, skills on graphing and statistical software were self-taught.

7.10 Future work

There are many ways in which the investigation could be enhanced if further work was to take place. One of the questions left unanswered is the composition of the water conditioner. As no enzymes nor protein were detected, the factor causing the clumping or VBNC formation remains unknown and of great interest.

Another future experiment of importance would be to identify the reactive species present in the active oxygen tablets and confirm whether pentapotassium peroxymonosulfate (PMS) is part of the product formulation, as this was supposed to be a part of this project and was not performed due to lack of access to chemical engineering labs.

Regarding pH experiments, there are several ways in which the investigation could be enhanced. Initially, a wider range of pH could be assessed. It was shown in this project that even small pH changes can have a large effect on planktonic growth and biofilm formation, and the addition of a wider range of pH could provide interesting results. In addition, the pH of adjusted cultures could be measured after overnight incubation, as well as after product addition.

In respect to potential VBNC formation, experiments could be performed to determine whether those cells could resuscitate in hot tub conditions, including water change or sub-MIC of products, as this could potentially lead to medical complications. In addition, as the drawbacks of SYTO 9/PI staining have been described, p-iodonitrotetrazolium violet (INT)

staining could also be performed to detect metabolically active VBNC (Schottroff et al., 2018). Screening for SCVs could also be performed.

In addition, the generation of more realistic experimental conditions would also be interesting. For example, the efficacy of the products could be assessed, in addition to growth media, in real hot tub water samples or swimming pool water. It may be possible to ask the sponsoring company to provide a sample, or set up a microcosm experiment as it has been already performed in some studies in order to perform studies of the aquatic environment (Mauro et al., 2013). Besides a series of factors such as temperature and water movement could be closely monitored and adjusted to provide an accurate representation of the hot tub environment. For example, the initial temperature could start at 25^{o}C, or a more realistic environmental temperature, and then slowly heat up to 37^{o}C, mimicking the heat up process of a hot tub. In addition, experiments utilizing temperature fluctuations could be performed, by heating up and allowing the media to reach room temperature for a few cycles. This could potentially provide useful information regarding the stability of Active Oxygen under repeated temperature fluctuations that take place in the hot tub environment.

Furthermore, confocal microscopy of the biofilm structures using cell viability staining could be performed (Cerca et al., 2012). It could also be useful for observing the effects that the products may have upon the biofilm structure (Azeredo et al., 2016).

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